Illinois U Library

VOLUME 42 [J. CELL. AND COMP. PHYSIOL.] NUMBER 3

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

BOARD OF EDITORS

DETLEV W. BRONK, Managing Editor
The Johns Hopkins University

W. R. AMBERSON
University of Maryland

M. H. JACOBS
University of Pennsylvania

A. C. REDFIELD
Harvard University

E. NEWTON HARVEY
Princeton University

E. K. MARSHALL, JR.
The Johns Hopkins University

H. W. SMITH
New York University

L. IRVING
Arctic Health Research Center

G. H. PARKER
Harvard University

RAYMOND E. ZIRKLE
The University of Chicago

DECEMBER 1953

PUBLISHED BIMONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
WOODLAND AVENUE AND THIRTY-SIXTH STREET, PHILADELPHIA 4, PA.

Entered as second-class matter February 19, 1932, at the post office at Philadelphia, Pa., under Act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in section 1103, Act of October 3, 1917, authorized on July 2, 1918

Price, $7.50 per volume, Domestic; $8.00 per volume, Foreign

Has YOUR Subscription Expired?

This is the last number of

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

to be published in 1953, and with it most subscriptions expire.

If you have not already renewed, do so now, so that 1954 numbers as published will reach you promptly.

Send subscriptions direct or through your agent to

THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
Woodland Avenue and 36th Street
Philadelphia 4, Pa.

RADIATION DOSE REDUCTION BY CYSTEINE

H. M. PATT, S. H. MAYER, R. L. STRAUBE
AND E. M. JACKSON
*Division of Biological and Medical Research, Argonne
National Laboratory, Lemont, Ill.*

SIX FIGURES

Cysteine has been shown to diminish many of the biological effects of x-irradiation, e.g., lethality of bacteria (Forssberg, '50; Hollaender, Stapleton and Burnett, '51), isolated cells Patt, Blackford and Straube, '52), tissues (Hall, '52), and animals (Patt et al., '49, '50; Smith et al., '50); epilation (Forssberg, '50); greying of hair (Kulwin, '52); lenticular opacities (Von Sallmann, '52); leucopenia and anemia (Patt, Smith and Jackson, '50). Thus, it would appear that cysteine may raise the threshold for x-radiation effects generally, with the probable exception of those attributable to direct ionization or excitation. Hypoxia (Thoday and Read, '47; Dowdy, Bennett and Chastain, '50) probably belongs in the same category in contrast with more specific procedures such as estrogen pretreatment (Treadwell, Gardner and Lawrence, '43; Patt et al., '49), spleen shielding (Jacobson et al., '49, '50), and bone marrow injection (Lorenz et al., '51). The present experiments were designed to determine primarily whether (1) cysteine results in a uniform dose reduction for several radiobiological effects in the mammal, i.e., lethality, lymphopenia, granulocytopenia, and splenic involution and (2) dose reduction consists, in effect, of the deletion of a fixed or proportional amount of the radiation.

METHODS

Female CF no. 1 mice, 10 to 12 weeks of age and weighing 19 to 25 gm, were used in these studies. Animals were main-

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY, VOL. 42, NO. 3
DECEMBER 1953

tained on a diet of Derwood Checkers and water ad libitum and were caged in groups of 10 to 15, each cage containing representatives of the various experimental groups. Mice were irradiated in pairs. One member of each pair received cysteine by tail vein injection immediately prior to the exposure; in some experiments, however, cysteine was administered after a fraction of the radiation dose was given. Some of the control mice were injected with an equivalent volume of 5% sodium chloride, but this practice was discontinued as the experiments progressed, when it was ascertained that there was no significant difference in radiation sensitivity between saline-injected and non-injected mice. Cysteine was administered as a 12.5% solution of the hydrochloride neutralized to pH 7 with 10 N sodium hydroxide. Only freshly prepared solutions were employed; these were discarded after 30 to 60 minutes when a faint cloudiness, indicative of beginning oxidation, was apparent. The cysteine dose ranged from 300 to 1200 mg/kg of body weight. The irradiation consisted of a total-body exposure to x rays with the mice placed in individual cellulose acetate exposure cells. The radiation factors were 250 kv, 15 ma, 0.5 mm Cu and 3.0 mm Bakelite filters, 26.5 cm target distance, 1.5 mm Cu half-value layer, and 220 r per minute dose rate. Total dosages of 400 to 1000 r were used. The various types of experiments performed are considered under the following headings.

Lethal dose studies. A split-exposure technique in which cysteine was injected after a fraction of the radiation dose was delivered was employed in some experiments. The interval between successive radiation fractions did not exceed 5 minutes. The pre- and post-cysteine fractions varied from 100 to 700 r. In other experiments the cysteine dose was also fractionated, a proportional amount preceding each radiation fraction. For example, 600 mg/kg were injected prior to an exposure of 400 r and this was followed immediately by a second injection of 600 mg/kg and a second exposure to 400 r. Other cysteine and radiation proportions were included in this series.

The effective roentgens received by cysteine-treated mice were read from a simultaneously determined dose-mortality curve for untreated mice. The dose reduction in the case of the split-exposure procedure with a single intervening cysteine injection was computed in the following manner on the assumptions that cysteine did not protect against the radiation fraction preceding it and that partial irradiation did not influence the protection afforded against subsequent exposure.

$$\begin{array}{c}\text{Dose reduction in roentgens}\\ \text{(post-cysteine exposure)}\end{array} = (r_2) - (\text{effective } r - r_1)$$

where effective r = roentgens required for equivalent control mortality

r_1 = roentgens first radiation fraction

r_2 = roentgens second radiation fraction

When the dosages of cysteine and x-irradiation were both fractionated, the effective roentgens derived from the control dose-mortality curve were compared with theoretical values, which were calculated in the following manner:

$$\text{Theoretical effective roentgens} = (r_1 - k_1 r_1) + (r_2 - k_2 r_2)$$

where k_1 = dose reduction constant previously derived for the given cysteine fraction

k_2 = dose reduction constant previously derived for the total cysteine dose.

In this instance, it is assumed further that the total cysteine dose protects against the second radiation fraction. The validity of these assumptions will be considered in the discussion.

Peripheral blood count and spleen weight. In these studies, mice were injected with cysteine (1200 mg/kg I.V.) immediately prior to total-body x-irradiation with doses ranging from 50 to 500 r. Irradiated control mice received an equivalent volume of 5% sodium chloride. The radiation factors were identical with those noted above. Blood sampling was accomplished by making a deep cut in the tail with a sharp razor and using a free flow of blood. Determinations were made three days after irradiation and included the total and differential leucocyte counts. The mice were then sacrificed with nembutal, the spleens and the left kidneys removed, dissected free of fat and weighed on a torsion balance to the nearest 0.1 mg. Kidneys were included as a control. Similar data were obtained for non-irradiated cysteine-injected and saline-injected mice.

RESULTS

For purposes of orientation, the radiation dose-mortality curve for untreated CF no. 1 female mice weighing 19 to 25 gm is presented in figure 1. The curve, which was derived from the probit least square line, reveals a 30-day LD_{50} of 630 r. The linear relationship between the dose reduction induced by cysteine and the dose of radiation is shown in figure 2, where it will be noted that a given amount of the amino acid (1200 mg/kg) accounts for 60 to 365 r depending upon the magnitude of the post-cysteine radiation fraction. The line was derived by the least square method; its slope indicates that 1200 mg of cysteine per kilogram cancels about 42% of the lethal action regardless of the intensity of exposure, at least over the range of 100 to 1000 r.

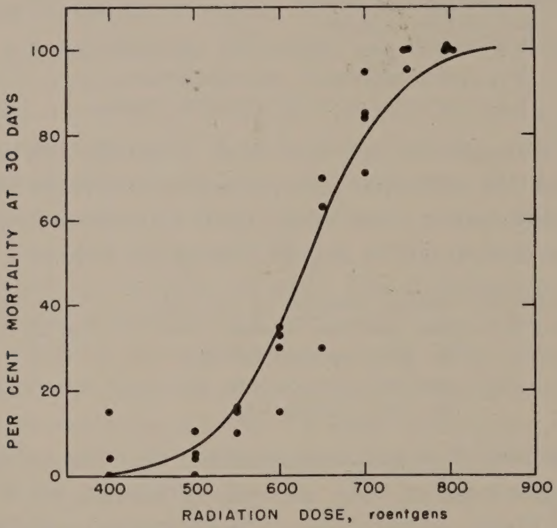


Fig. 1 Dose-mortality curve for CF no. 1 female mice. (Each point represents the 30-day mortality of a group of 20 mice.)

The effect of various concentrations of cysteine is presented in figure 3. The minimal effective dose is in the neighborhood of 200 mg/kg and the per cent dose reduction apparently increases as a linear function of the log of the cysteine dose.

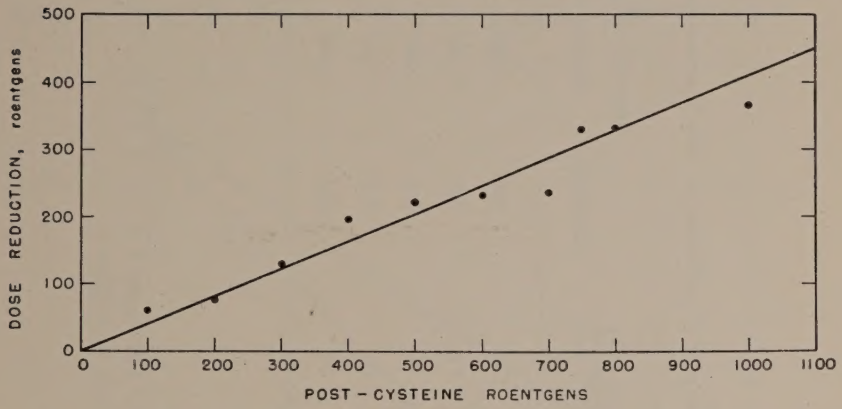


Fig. 2 Relationship between the dosage of x-irradiation and the protection against lethality afforded by 1200 mg cysteine per kilogram. (The line is derived by the least squares method; each point represents a group of 20 mice.)

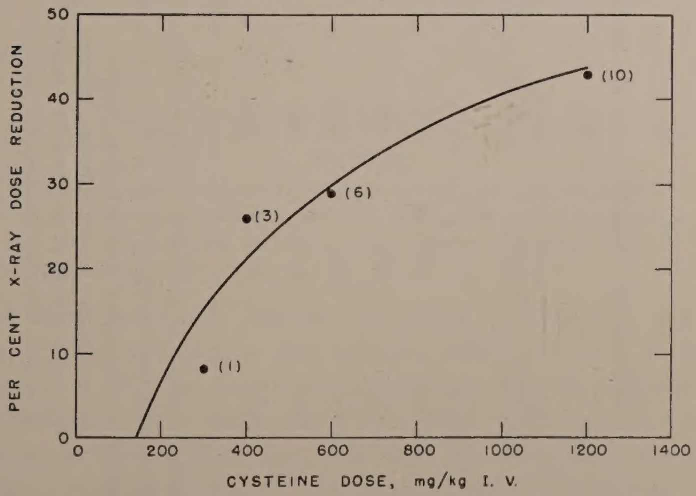


Fig 3 Radiation dose reduction as a function of cysteine dosage. (The criterion is 30-day lethality in the mouse; figures in parentheses refer to number of groups of 20 mice each upon which mean value is based.)

TABLE 1

Radiation dose reduction when the dosages of cysteine and X radiation are both fractionated

NO. MICE	FIRST CYSTEINE FRACTION	FIRST RADIATION FRACTION	SECOND CYSTEINE FRACTION	SECOND RADIATION FRACTION	PER CENT MORTALITY AT 30 DAYS	EFFECTIVE ROENTGENS		
						Determined [1]	Theoretical [2]	Per cent deviation from theoretical
	mg/kg	*r*	*mg/kg*	*r*				
24	150	100	1050	700	8	500	492	1.6
44	300	200	900	600	14	528	506	4.4
63	600	400	600	400	8	500	504	0.8
20	600	450	600	450	40	610	567	7.6
20	600	500	600	500	70	660	630	4.8

[1] Roentgens required for equivalent control mortality.

[2] Theoretical effective roentgens = $(r_1 - k_1r_1) + (r_2 - k_2r_2)$

where r_1 = roentgens first radiation fraction
r_2 = roentgens second radiation fraction
k_1 = dose reduction constant for cysteine fraction derived from figure 3
k_2 = dose reduction constant for total cysteine dose derived from figure 3.

Twelve hundred milligram cysteine per kilogram is the maximally tolerated dose when given as a single intravenous injection. When the dosages of cysteine and x-radiation are both fractionated, the effective roentgens computed from the 30-day mortality are similar to the values predicted from the data in figure 3. These results are summarized in table 1.

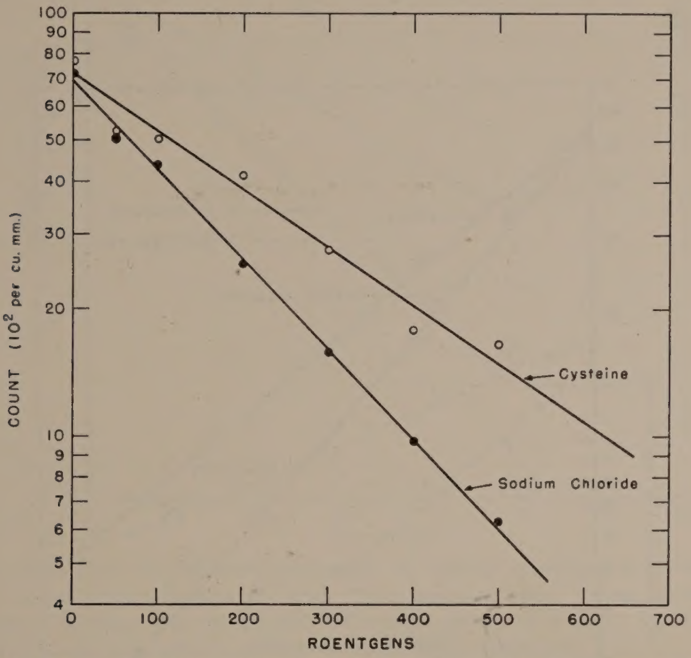


Fig. 4 Effect of cysteine pretreatment on radiation-induced leucopenia. (Points at 50 to 300 r represent mean values derived from groups of at least 15 mice, those at 400 and 500 r from groups of 8 mice. The difference between slopes is statistically significant, $p < .01$.)

The dose-response relationships for leucopenia, lymphopenia, granulocytopenia, and splenic involution are presented in figures 4–6. The proportional nature of the protection is readily apparent in each case. Differences between the slopes of the least square lines derived from the cysteine treated and control irradiated groups are statistically significant ($p < .01$). Kidney weights were not altered appreciably in either of the irradiated groups. Moreover, cysteine per se did not affect the peripheral blood picture nor the weight of the spleen or kidney. The dose reduction for leucopenia, lymphopenia, and involution of the spleen is rather similar to that observed for lethality; it appears to be somewhat greater ($p < .05$), however, for granulocytopenia. The comparative protection by cysteine for the various biological effects is indicated in table 2.

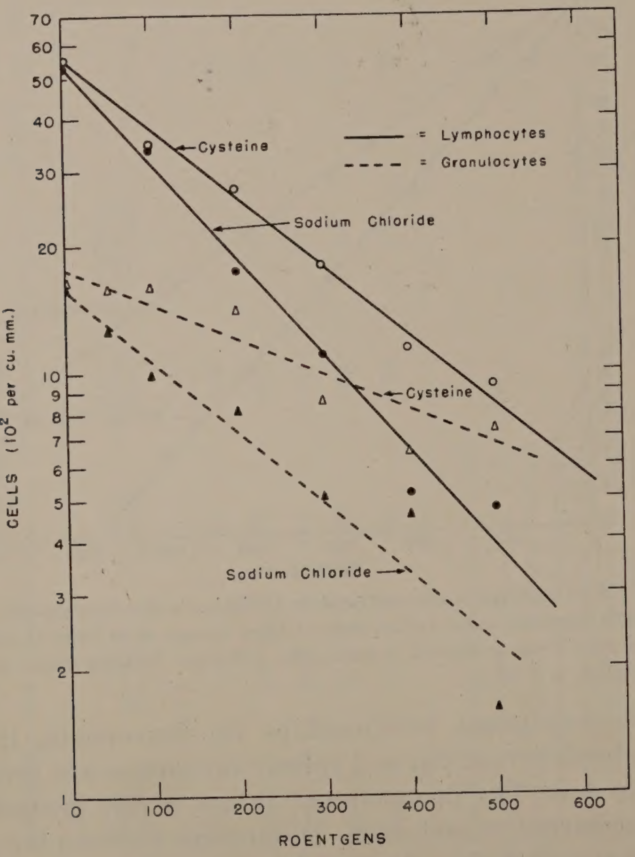


Fig. 5 Effect of cysteine pretreatment on radiation-induced lymphopenia and granulocytopenia. (Points at 50 to 300 r represent mean values derived from groups of at least 15 mice, those at 400 and 500 r from groups of 8 mice. The difference between slopes is statistically significant, $p < .01$.)

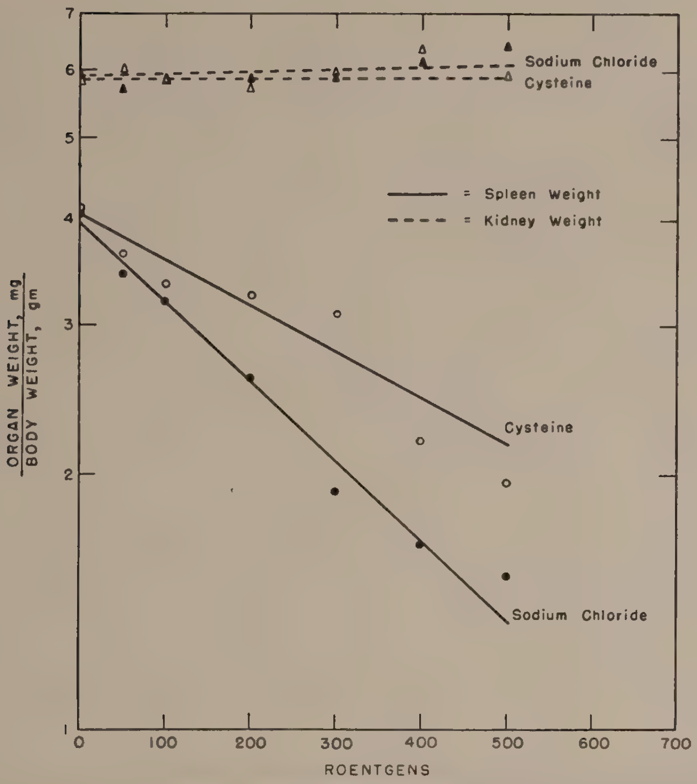


Fig. 6 Effect of cysteine pretreatment on splenic involution following total-body x-irradiation. (Points at 50 to 300 r represent mean values derived from groups of 20 mice, those at 400 and 500 r from groups of 8 mice. The difference between slopes for splenic atrophy is statistically significant, $p < .01$.)

TABLE 2

Comparative protection with cysteine against several effects in X-irradiated mice (Cysteine dose = 1200 mg/kg)

BIOLOGICAL EFFECT	DOSE RANGE r (total-body X radiation)	PER CENT DOSE REDUCTION
Lethality [1]	100–1000	42.0 ± 6.9 [2]
Leucopenia	50– 500	37.4 ± 3.9
Granulocytopenia	50– 500	56.2 ± 6.8
Lymphopenia	50– 500	31.3 ± 4.9
Splenic involution	50– 500	42.3 ± 6.7

[1] Cysteine injected after a fraction of the total radiation dose was delivered.

[2] Standard error.

DISCUSSION

It is well known that curves relating mortality to the radiation dose are steep and that large variations in mortality may occur in the LD_{50} range. The importance of employing a wide spectrum of radiation doses in order to evaluate the efficiency of a protective or potentiating agent is, therefore, self evident. The split-exposure procedure, in which a standard dosage of cysteine is given between two radiation fractions[1] or a proportional amount of cysteine precedes each fraction, not only permits a more precise evaluation of the nature of the protection but may also circumvent the obvious limitation imposed upon any evaluation in which lethality is the criterion. It is possible, moreover, by proper selection of the radiation fractions to approach the plateau of the dose-mortality curve and thus to decrease the inherent variability of the lethal effect. The validity of the split-exposure procedure for such purposes is based upon the several premises already alluded to under Methods (p. 329) and cannot be determined a priori for a specific agent. However, the linearity of the protection whether cysteine injection is preceded or succeeded by 100 to 700 r (fig. 2) and the close agreement between the determined and theoretical effective roentgens when the dosages of cysteine and radiation are both fractionated (table 1) argue for the validity of these assumptions in the case of cysteine. These findings indicate that irradiation just prior to cysteine administration does not influence the protection against subsequent exposure and that the lethal potential of small dosages of radiation cannot be reversed by cysteine under these conditions. It is also apparent that two suitably spaced cysteine injections are additive in their protective effect. These results may be of some practical as well as of theoretical interest.

The data demonstrate clearly that the dose reduction attendant upon cysteine pretreatment consist of the deletion of a proportional amount of the radiation over a wide dose range, at least for the several radiobiological effects that have been studied. A similar proportionality has been observed with thymic cells (Patt, Blackford and Straube, '52). It is apparent, moreover, that cysteine affords a relatively uniform protection against radiation lethality, splenic involution, leucopenia, lymphopenia, and granulocytopenia. The differences with exception of granulocytopenia are not significant statistically. The significance of the slightly greater protection of granulocytes ($p < .05$) does not compromise seriously the homogeneity of the protection, especially in view of the inherent complexity of the physiological situation. It is possible that the relative recovery rates for the different effects may be influenced preferentially by cysteine (recovery is ordinarily seen first in granulocytes, then in lymphocytes), or that other factors secondary to irradiation may be involved. Peripheral blood counts and spleen weights were determined three days after x-irradiation. This interval was selected because it was considered that, in general, the maximal effects would be observed at this time with the dosage range used. It has been reported, moreover, that the correlation between thymic and splenic atrophy and radiation dosage is seen only during the first few days after exposure before appreciable recovery occurs (Carter, Harris and Brennan, '50).

---

[1] In a personal communication, Friedell and Salerno have indicated their use of a similar technique in which cysteine was given between two intervening exposures. Their findings are essentially comparable to those reported here.

The degree of protection is dependent upon cysteine concentration and appears to increase linearly as the log of the cysteine dose. The significance of this relationship is not clear. Each dose level results in a fairly constant percentage dose reduction, at least in terms of 30-day lethality, which is independent of the radiation dose. These findings amplify earlier work (Smith et al., '50) on the protection against lethality in x-irradiated rats, in which various cysteine doses were employed at a given level of irradiation and vice versa.

In view of the ubiquitous nature of the energy absorption and the complexity of the responding organism, it is perhaps not surprising that many kinds of radiation effects are observed and that a number of factors are capable of modifying them. While some of the modifying factors are concerned with the events responsible for injury to specific physiological systems or for recovery from such injury, e.g., spleen shielding and bone marrow injections, others exert a more primary and apparently nonspecific influence against certain of the immediate radiation reactions. Uniformity of the protection by cysteine against a number of radiation sequelae in the intact animal points to a true dose reduction in the sense that it alters a common pathway. This implies that the decisive action occurs at an early stage in the chain of events, possibly by altering the pathways of energy dissipation, e.g., activated water reactions or the nature of the biological receptors, e.g., enzymes, substrates or other molecules essential for structural and functional integrity. This does not imply protection against all radiation changes insofar as different primary mechanisms may be involved in their development. There is reason to believe from studies with tumor fragments (Hall, '52) and packed cells (Patt, Blackford and Straube, '52) that cysteine probably acts at the level of the cell, although whether on the cell surface or intracellularly cannot yet be stated. It is well to recall, however, that an extracellular site of action may be of considerable importance in a relatively uncontaminated cell suspension, e.g., bacteria suspended in phosphate buffer (Hollaender, Stapleton and Burnett, '51).

While the dose reduction induced by cysteine could be explained simply by an increase in the absorbing material for the oxidants presumably formed in intracellular water, or by a decrease in the availability of intracellular oxygen and hence in the yield of certain of the oxidants (e.g., HO_2), there are indications that metabolic pathways may also be involved in some of the protection effects or at least that the protective phenomenon cannot be interpreted completely in terms of immediate oxidative reactions. Protection of thymic cells by cysteine is dependent upon temperature both before and during the early post-irradiation period (Patt, Blackford and Straube, '52). Thymic cells (Patt, Blackford and Straube, '52) and onion epidermis cells (Bellack and Krebs, '51) are also partially protected when the amino acid is added immediately after x-irradiation. Cysteine has been shown to reduce the rate of x-ray inactivation of phage T2H suspended in nutrient broth or in a gelatine solution (Doermann, '51). It is not known, however, whether the protective action in the presence of broth indicates a direct effect of cysteine on the virus particles or an interaction of cysteine with toxic agents produced in the water of hydration. That the protection may have a biological as well as a radiochemical basis is perhaps also suggested by the greater efficiency of cysteine protection against acute lethality in abdomen-shielded than in abdomen-irradiated rats (Swift, Taketa and Bond, '52). The proportional nature of the dose reduction is not inconsistent with either postulate.[2]

SUMMARY

Cysteine has been shown to afford a relatively uniform protection against a number of x-radiation sequelae in the mouse (i.e., lethality, splenic atrophy, leucopenia, lymphopenia, and granulocytopenia). This implies that the protective action occurs at an early stage in the chain of events induced by irradiation and points to a true dose reduction. The proportional nature of the protection, i.e., deletion of a constant percentage of the biological effect which is independent of radiation dosage, has been observed for the various effects studied. The degree of protection against radiation lethality appears to increase linearly as the log of the cysteine dose. The results obtained with a split-exposure procedure, in which a standard dosage of cysteine was given between two radiation fractions or a proportional amount preceded each fraction, suggest that (1) irradiation just

---

[2] Recent experiments by Patt, Clark, and Vogel reveal that differences in the magnitude of protection by cysteine against the lethal effects of gamma and fast neutron irradiation in mice are in essential agreement with the inverse relationship between ionization density and the extent of the oxygen effect. For the present, cysteine protection in the intact animal may be interpreted best in terms of the spatial distribution of the radicals formed in water and their reactions.

prior to cysteine administration does not influence the protection against subsequent exposure; (2) the lethal potential of small dosages of radiation cannot be reversed by cysteine under these conditions; and (3) the protective action of two suitably spaced cysteine injections is additive. The possibility that the modifying influence of cysteine may have a biological as well as a radiochemical basis has been considered.

ACKNOWLEDGMENT

The authors greatfully acknowledge the assistance of Joan Gurian and Sylvanus Tyler in the statistical analysis of the data.

LITERATURE CITED

BELLACK, S., AND A. T. KREBS 1951 Protection of single cells and small cell groups against radiation. Army Medical Research Laboratory Report No. 6–64–12–06–(43).[3]

CARTER, R. E., P. S. HARRIS AND J. T. BRENNAN 1950 The effect of acute doses of X-irradiation on the splenic and thymic weight of CF-1 female mice. U. S. Atomic Energy Commission Document, LA-1075.[3]

DOERMANN, A. H. 1951 X-ray sensitivity of bacteriophage T2H in the presence of various selected chemical compounds. ORNL-1026, p. 20.[3]

DOWDY, A. H., L. R. BENNETT AND S. M. CHASTAIN 1950 Protective action of anoxic anoxia against total body roentgen irradiation of mammals. Radiology, *55:* 879–885.

FORSSBERG, A. 1950 On the possibility of protecting the living organism against roentgen rays by chemical means. Acta Radiol., *33:* 296–304.

HALL, B. V. 1952 Growth of tumor fragments X-irradiated *in vitro* following pretreatment with cysteine. Cancer Research, *12:* 787–792.

HOLLAENDER, A., G. E. STAPLETON AND W. T. BURNETT, JR. 1951 The Modification of X-ray Sensitivity by Chemicals. Isotopes in Biochemistry, A Ciba Foundation Conference. Churchill Ltd., London, pp. 96–113.

JACOBSON, L. O., E. K. MARKS, M. J. ROBSON, E. O. GASTON AND R. E. ZIRKLE 1949 The effect of spleen protection on mortality following X-irradiation. J. Lab. Clin. Med., *34:* 1538–1543.

JACOBSON, L. O., E. L. SIMMONS, E. K. MARKS, M. J. ROBSON, W. F. BETHARD AND E. O. GASTON 1950 The role of the spleen in radiation injury and recovery. J. Lab. Clin. Med., *35:* 746–770.

KULWIN, M. H. 1952 Personal communication.

LORENZ, E., D. UPHOFF, T. R. REID AND E. SHELTON 1951 Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J. Nat. Cancer Inst., *12:* 197–201.

---

[3] Information regarding the availability of these reports may be obtained by addressing requests to the Office of Technical Services, Department of Commerce, Washington, D. C.

PATT, H. M., M. E. BLACKFORD AND R. L. STRAUBE 1952 Effect of X-rays on thymocytes and its modification by cysteine. Proc. Soc. Exp. Biol. Med., *80:* 92–97.

PATT, H. M., D. E. SMITH AND E. JACKSON 1950 The effect of cysteine on the peripheral blood of the irradiated rat. Blood, *5:* 758–763.

PATT, H. M., D. E. SMITH, E. B. TYREE AND R. L. STRAUBE 1950 Further studies on modification of sensitivity to X-rays by cysteine. Proc. Soc. Exp. Biol. Med., *73:* 18–21.

PATT, H. M., R. L. STRAUBE, E. B. TYREE, M. N. SWIFT AND D. E. SMITH 1949 Influence of estrogens on the acute X-irradiation syndrome. Am. J. Physiol., *159:* 269–280.

PATT, H. M., E. B. TYREE, R. L. STRAUBE AND D. E. SMITH 1949 Cysteine protection against X irradiation. Science, *110:*. 213–214.

SMITH, D. E., H. M. PATT, E. B. TYREE AND R. L. STRAUBE 1950 Quantitative aspects of the protective action of cysteine against X-radiation. Proc. Soc. Exp. Biol. Med., *73:* 198–200.

SWIFT, M. N., S. T. TAKETA AND V. P. BOND 1952 Pretreatment with cysteine in partial body X-irradiation. Fed. Proc., *11:* 158.

THODAY, J. M., AND J. READ 1947 Effect of oxygen on the frequency of chromosome aberrations produced by X ray. Nature, *160:* 608–610.

TREADWELL, A. DE G., W. U. GARDNER AND J. H. LAWRENCE 1943 Effect of combining estrogen with lethal doses of roentgen-ray in Swiss mice. Endocrinology, *32:* 161.

VON SALLMANN, L. 1952 Further efforts to influence X-ray cataract by chemical agents. Trans. Am. Ophthal. Soc., *49:* 391–417.

THE NORMAL MEMBRANE RESTING POTENTIAL OF MAMMALIAN SKELETAL MUSCLE MEASURED IN VIVO

A. L. BENNETT, F. WARE, Jr., A. L. DUNN AND A. R. McINTYRE
Department of Physiology and Pharmacology, University of Nebraska, College of Medicine, Omaha

TWO FIGURES

The technique for direct measurement of the difference in potential between the inside and outside of single muscle cells was provided by Graham and Gerard ('46) when they first penetrated frog muscle cells with micro-electrodes consisting of extremely fine glass capillaries containing saline. Ling and Gerard ('49) described the preparation and use of such electrodes with a tip-diameter of less than 1 μ and presented preliminary experiments in which the electrodes were filled with 3 molar instead of isotonic KCl. These authors and also Nastuk and Hodgkin ('50) have reported detailed studies on the resting and action potentials of frog muscle fibers. The same technique has been used by Nastuk ('50, '51) and Fatt and Katz ('51, '52) to study resting and action potentials at the myoneural junction in frog muscle fibers. The technique has been extended to the study of other excised tissues; frog heart muscle (Woodbury and Hecht, '50; Woodbury, Hecht and Christopherson, '51; Dunn, Bennett and McIntyre, '52); mammalian heart muscle (Draper and Weidman, '51); embryonic myocardium (Fingle, Woodbury and Hecht, '52); frog and mammalian nerve fibers (Woodbury and Woodbury, '50; Woodbury, '52); giant axons of Sepia (Weidman, '51); frog dorsal root ganglion cells (Svaetichin, '51). Recently Brock, Coombs and Eccles ('52) have reported use of the technique on mammalian nerve tissue *in vivo,* e.g. motor cell bodies within the cord of the cat. Hoffman and Suckling ('52) have used the method in the study of cellular potentials of intact mammalian hearts.

The present paper is a detailed report of the measurement of the membrane resting potential of skeletal muscle cells in the anesthetized mouse by the glass micro-electrode technique. A preliminary report on resting potentials of normal and denervated mammalian skeletal muscle, measured *in vivo,* was made by Ware, Bennett and McIntyre ('52a, b).

METHOD

Capillary electrodes. Capillary electrodes with a tip-diameter of less than 1 μ can be drawn by hand in a miniature flame as described by Ling and Gerard. We have found a micro-forge with automatic spring pullers a great help in the rapid and consistent drawing of electrodes and we present here a brief description of how it was made and the procedure in its use. Dimensions are not critical and anyone wishing to duplicate it could easily do as we have done with parts from discarded apparatus. With this micro-forge we have found it possible to draw capillaries with a tip-diameter of 0.5 μ or less. Over half of the attempts yield electrodes with a tip-diameter of 1 μ or less. Ten such usable electrodes have been made in less than one hour. Unsatisfactory tips can be quickly redrawn on the same shank. Short or long tapers may be drawn at will. The electrodes were filled with 3 molar KCl. The microscopic portion of the tube filled by capillary attraction in two or three days while the electrodes were stored in the KCl solution. The shank was then filled through a length of 27 gauge hypodermic tubing.

Capillary electrode micro-forge. Figure 1 is a diagram of the essential parts. The support consists of the base of an old Duboscq-type colorimeter (Co.) with its two cup supports on vertical rack adjustments. This base is conveniently tipped toward the operator at a 45° angle. To one cup support is bolted an old microscope mechanical stage (M.S.). To the

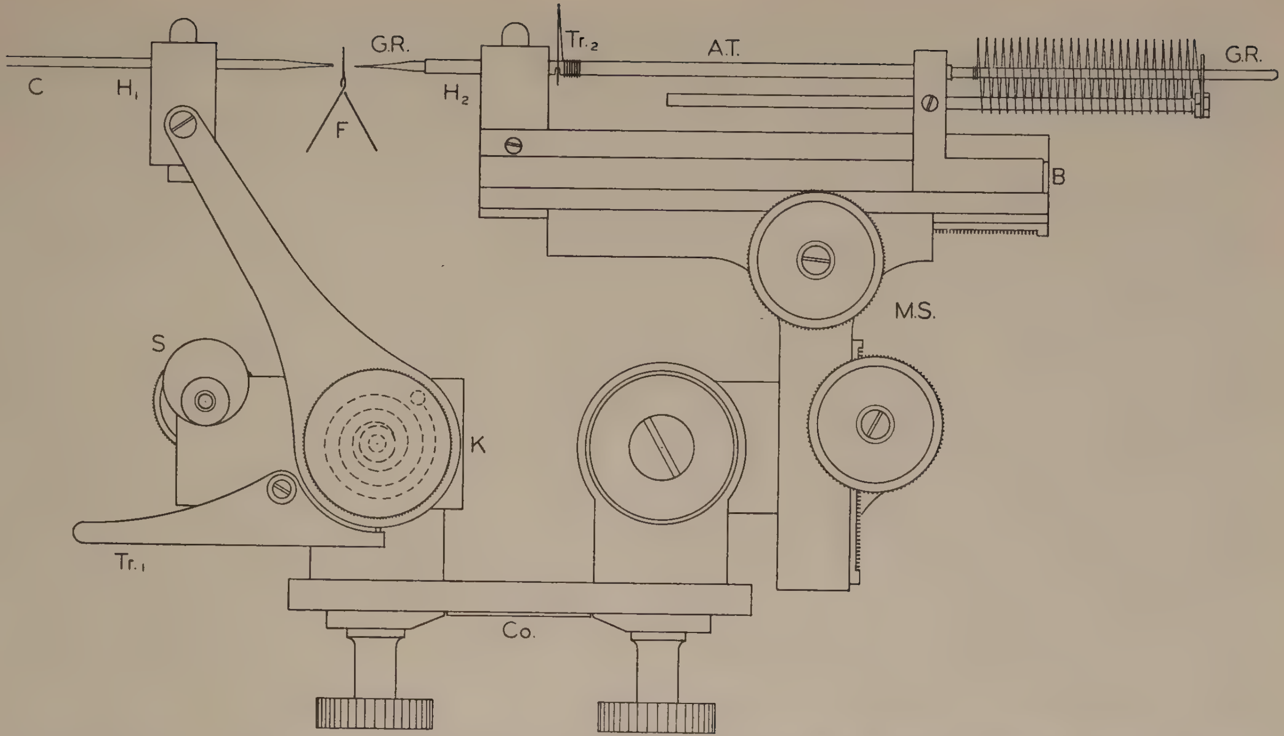


Fig. 1 Diagram of electrode micro-forge made from old parts. Co., colorimeter base; M.S., mechanical stage; K., spring-tension-control knob on pivoted arm; S., adjustable stop; H.$_1$ and H.$_2$, spring-clip holders; C., capillary tube; G.R., glass rod; A.T., aluminum tube; B., sliding bracket; Tr.$_1$ and Tr.$_2$, trigger releases for springs; F., hot-wire filament. Further details in text.

other cup support is bolted a plate carrying an arm, pivoted on the shaft of knob (K.). This knob adjusts the spring tension which swings the arm to the left. On the end of the arm is the spring-clip holder ($H._1$) for the small glass tube (C.) which is to be drawn to capillary size. These tubes (approximately 2 mm in diameter) have been previously drawn to a tip-diameter of about 0.5 mm in a flame. The trigger ($Tr._1$) releases this holder as the handle is pushed, thus withdrawing the pin from a hole in the edge of the arm.

At the left end of the mechanical stage a second spring-clip holder ($H._2$) grips an aluminum tube (A.T.) which is rigidly fastened to the sliding bracket (B.). A set screw in this bracket grips the brass rod which supports the coil spring at the right together with a loose guide for the glass rod (G.R.). The left end of the coil spring grips the glass rod. Tension of this spring is adjusted by sliding the brass rod in the bracket. The trigger ($Tr._2$) which releases this spring for movement of the glass rod to the right consists of a coil of spring bronze wire the long end of which is sprung into a notch in the aluminum tube where it engages a small groove cut in the glass rod. By a flick of the finger against the wire loop the coil is easily twisted on the tube to raise the end of the wire from the notch and thus release the rod.

The hot-wire filament (F.) consists of a 4 mm loop of 24 gauge (0.5 mm) nichrome wire. The approximately 30 watts required to heat it is conveniently supplied by a heavy duty transformer such as that in a Little Giant Electric Soldering Machine with a foot switch.

A binocular dissection microscope, supported over the micro-forge at a 45° angle and a microscope lamp mounted beyond the hot-wire filament complete the set-up.

Procedure for drawing capillary electrodes. With the two triggers locked, the glass rod and tube are fused within the nichrome loop. The spring holder on the glass tubing is slackened and the glass displaced a millimeter or two to the right. The tube is softened and drawn manually with the mechanical stage to a diameter of approximately 30 μ if a short taper is desired. The larger the diameter at this point, the longer the taper will be. Both triggers are released, the spring tensions having been previously adjusted to balance. Slight manual adjustment of the now freely movable glass places the desired portion of the capillary within the wire loop. While the opposing spring tensions are maintained, the heat is turned on and the final drawing is automatically accomplished. The low inertia system at the right withdraws rapidly to shrink the diameter of the glass. The heavier holder at the left maintains the position of the capillary in the heated loop and then withdraws the tip before it curls.

By releasing the spring clip of holder ($H._2$) and sliding out the bracket (B.), the spring and tube assembly may be removed. Glass rods may then be put in each holder for the drawing and forming of micro-dissection tools.

Potentiometer equipment. Silver-silver chloride electrodes were used in the shank of the capillary and in a well at the end of a KCl-agar bridge which dipped into the Tyrode's solution to form the indifferent electrode. Both electrodes were filled with 3 molar KCl.

The cathode-follower preamplifier was similar to those used by other workers. Victoreen tubes, no. 5803, limited grid current to 10^{-14} amperes. An input switch (steatite insulation) made it convenient to introduce a shunt of known resistance for the purpose of measuring the resistance of the microelectrodes.

To obviate the drift inherent in D-C amplifiers a conventional push-pull, resistance-capacity coupled amplifier was used with a chopper (120 cps) in parallel with the input. The time constant of the amplifier was 0.8 seconds. The C-R oscilloscope was used as a null-point indicator which registered the square wave produced by the chopper whenever the potential at the tissue was not balanced by the calibrator which supplied the bucking E.M.F. between the indifferent electrode and ground. A loud speaker, loosely coupled through approximately 50 mmfd to the output of the amplifier provided a low intensity cracking signal of imbalance to aid in the placement of the micro-electrode while using the microscope. Upon penetration of a cell with the micro-electrode, balance with the calibrator and reading of the meter could be accomplished with an accuracy of ± 1.0 mV. within 5 seconds.

Procedure. White mice, weighing 16–26 gm, were anesthetized with an oxygen-cyclopropane mixture. A special microgauge on the two gas lines made possible smooth control of the anesthetic. Oxygen at 300 cm^3 per minute and cyclopropane at 50–100 cm^3 per minute flowed through a small glass chamber closed around the neck of the mouse with a rubber diaphragm. The skin overlying the tibialis anterior was removed from the right leg with care to avoid bleeding from subcutaneous veins in the region. The mouse, on its back, was then placed with the hind quarters resting in a Petri dish through which Tyrode's solution continually circulated at controlled temperature. The right leg was held against the bottom of the Petri dish with two insulated spring holders, one across the femur and one on the foot. Micro-manipulation and observation were carried out with two Emerson micromanipulators and a binocular dissection microscope using reflected light.

Continuous circulation of the Tyrode's solution kept the operative field clear of any blood. In early experiments this solution was maintained at 37°C. However, the high ambient temperature of the room (often over 30°C. during the summer) plus the warm solution prevented adequate heat dissipation by the animal, resulting occasionally in death. For this reason, later experiments were done with the bath at room temperature ranging from 24–30°C. No correlation was observed between resting potential and bath temperature when making measurements on cells buried within the belly of the muscle.

Further dissection under magnification of 15 × cleared the muscle of subcutaneous areolar and fat tissue, and finally of the investing deep fascia. It was necessary to remove this deep fascia completely from all areas through which the micro-electrode was to be inserted. This latter step in the dissection was the most critical part of the preparation of the tissue. Many fine strands of connective tissue attach this fascia to superficial muscle fibers. These strands were severed with a sharp scalpel close to the muscle in order to obtain a clean surface with as little damage to muscle cells as possible. Proper dissection resulted in exposure of a clean, fusiform muscle whose individual fibers were easily seen using oblique, reflected light and a magnification of 45 ×.

As the muscle of the right leg was used, the micro-electrode, in the left manipulator at an angle of about 30° from horizontal, could be made to penetrate perpendicular to the curved surface of the muscle at a point where both the electrode and individual muscle fibers were mostly clearly visible. (An attachment for the Emerson manipulator has been made which makes possible parallel alignment of the manipulation rack and the electrode at various angles. This has been found to be of decided advantage for deep penetration of tissue and will be used in future experiments.)

Balance of the electrodes was checked every two to three minutes during an experiment with both electrodes free in the bath. Any imbalance usually remained constant within 1–2 mV. throughout an experiment. Failure to do so prompted immediate elimination of any leaks to ground or replacement of the electrode.

RESULTS

In 4 preliminary experiments the resting potential (R.P.) was measured in cells at the surface of the muscle where penetration of the electrode into individual cells could be carried out under direct observation. These values averaged about 83 mV. It was observed that in muscles which received a minimum of trauma during dissection the R.P. of surface cells was the highest. Specific cells which were seen to have suffered damage, as evidenced by cloudiness of the cytoplasm, had definitely lower potentials. Furthermore, as measurements were continued over a period of an hour or more, the average values steadily declined. These findings prompted measurements in protected cells by penetration with the electrode beyond the surface layer. It was immediately apparent that the R.P. values obtained from the deeper fibers averaged considerably higher than those from fibers on the surface of the muscle. Furthermore, the average of values obtained from deep fibers remained constant over several hours in contrast to the decline regularly seen when measurements were confined to the surface. Bathing the muscle with potassium-free Tyrode's solution resulted in a slight rise in the average R.P. of surface fibers but failed to influence the values obtained from the deeper fibers.

As a result of the above observations it was concluded that the relatively low and markedly variable potentials recorded from surface fibers resulted from damage. Some damage was unavoidable regardless of the care with which the dissection was carried out. Therefore, subsequent measurements were carried out on deep fibers, and very few values from surface fibers are included in the data presented.

By cautiously pushing the micro-electrode through the surface layer of cells and then progressively deeper within the muscle, it was possible to make 5 or 6 measurements with satisfactory penetration of individual cells. Since the penetration of each cell could not be visually controlled, the criteria for deciding that the electrode had entered and was properly sealed within a cell were based entirely upon the characteristics of the potential as measured. First, a sudden, marked increase in negativity of the micro-electrode as it was pushed slowly into the muscle was taken as a sign of abrupt penetration of a cell. This was in contrast to a more gradual rise of measured potential with progress of the electrode, characteristic of measurements giving relatively low and often unsteady values. Such penetrations were assumed to involve tearing or deforming effects at the membrane. Second, the potential remained steady rather than fluctuating, indicating that the cell had effectively sealed itself around the tip of the electrode. Third, the potential, instead of immediately and progressively falling, remained at or near its maximum value over a period of 10 to 15 seconds, or until movement of the electrode or muscle occurred. This was taken as a further indication that there was no leakage around the tip of the electrode. (Numerous measurements were continued for several minutes to demonstrate the steadiness of recorded potential with proper penetration of a cell.) Fourth, the potential was high rather than low. The use of such a criterion for judging proper penetration of a cell when actually carrying out the procedure for the purpose of measuring the potential obviously means arbitrary selection of data. Since all known sources of error were in a direction which would result in spuriously low rather than high values, no readings in the upper range were ignored. On the other hand, if a measurement indicated a low potential there was always a

TABLE 1

A summary of the fifteen experiments

DATE 1952	NUMBER VALUES	RANGE mV	MEDIAN mV	MEAN mV	STAN'D DEV.	STAN'D ERROR	COEFFIC'T VARIATION
							%
6/2	82	79–110	92.0	92.6	7.2	.80	7.8
6/3	60	88–110	100.0	98.7	5.3	.68	5.3
6/3	21	88–110	93.0	95.0	6.0	1.30	6.3
6/4	100	87–106	96.5	96.7	4.6	.46	4.8
6/5	111	86–115	100.0	99.4	6.7	.64	6.8
6/6	83	87–114	100.0	99.7	5.8	.64	5.8
6/9	105	88–116	102.0	102.9	6.2	.60	6.0
6/10	74	92–112	98.0	98.7	4.0	.47	4.1
6/12	82	91–115	99.0	99.3	4.8	.53	4.8
6/13	54	92–115	101.0	101.3	5.2	.71	5.1
6/16	106	90–123	104.5	104.9	9.4	.92	9.0
6/24	85	93–111	99.0	99.7	3.5	.38	3.5
6/25	79	91–113	101.0	100.9	4.6	.52	4.6
7/2	25	93–115	100.0	101.2	6.8	1.36	6.7
7/20	63	92–111	100.0	100.3	4.9	.62	4.9

Total number of measurements 1130, average 99.8 mV., standard deviation 6.5 mV., standard error of mean .19 mV.

Average of the 15 means 99.4 mV., standard deviation 3.0 mV., standard error of mean .78 mV.

Range of coefficients of variation 3.5% to 9.0% average coefficient of variation 5.2%.

question whether it represented a truly low R.P. in a normal cell, or whether it was the result of a faulty puncture of the cell or of other damage to the cell. As experience was gained in recognizing the range of values obtained in measurements which met the first three criteria, it became possible to make more intelligent decisions on this question. Very low potentials were ignored. A questionable value was recorded and then observed for 10–15 seconds. If the potential declined rapidly or showed pronounced unsteadiness, it was rejected. Otherwise it was included in the record. This procedure undoubtedly led to the omission of some low values which represented actual potentials in normal cells. The statistical analysis of the results as recorded is believed to indicate the essential validity of the criteria for inclusion of specific readings.

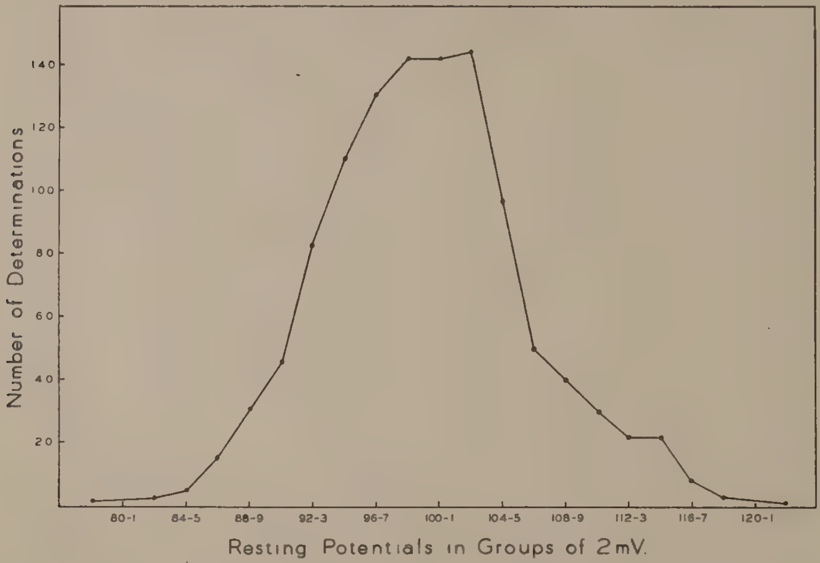


Fig. 2 Distribution curve for the 1130 measurements of R.P.

The results from a total of 1130 measurements made on 15 mice are listed in table 1. From 21 to 111 readings were taken on each animal. The spread of values from any one mouse ranged from 18 to 33 mV. The mean values for individual animals ranged from 92.6 mV. to 104.9 mV., averaging 99.4 mV. The standard deviation of this average of means was 3.0 mV.: thus if the means are normally distributed, 68% of them should fall within the range 96.4 to 102.4 mV. Actually the means in 11 of the 15 experiments, or 75%, did so. This was considered to indicate a good distribution of values and to vindicate the method of selection of low values where there was question of satisfactory puncture of the cells. The precision of the measurements, as indicated by the coefficients of variation in the individual experiments is considered satisfactory.

The highest R.P. observed was 123 mV., measured in the animal for which the mean was 104.9 mV. From this same animal one R.P. of 119 mV., two of 118 mV. and 5 of 117 mV. were recorded. The highest value obtained in any other animal was 116 mV. In 4 additional experiments the maximum R.P. was 115 mV.

A distribution curve for the entire series of 1130 measurements, irrespective of individual animals, is presented in figure 2. It is plotted in increments of 2 mV. The average of all the values is 99.8 mV. with a standard deviation of 6.5 mV. The standard error of the mean is 0.19 mV. Of all values 7.7% are 110 mV. or greater.

Since the mice were anesthetized with cyclopropane at the time measurements were made, there was the possibility that this agent, directly or indirectly, had affected the R.P., and that therefore the values obtained did not represent normal resting potentials. No differences in R.P. were observed when the anesthetic level was deliberately varied within wide limits on several animals. One experiment was performed to test the point more directly. After the usual preparation, 25 measurements were made with the animal under cyclopropane anesthesia. These averaged 101.2 mV. with a standard deviation of the mean of 1.36 mV. A paralyzing dose of d-tubocurarine was then injected intraperitoneally (15 cm^3 of a 1:100,000 solution per kilogram of mouse). Immediately following the injection, R.P. values of 99, 116 and 114 mV.

were obtained. Two minutes later the anesthetic was turned off. In the period from 9 to 15 minutes after the anesthetic was turned off, 12 measurements of R.P. averaged 99.3 mV. Anesthesia was then resumed and during the next 16 minutes 11 determinations gave R.P. values averaging 99.5 mV. Since mice recover consciousness within two minutes after withdrawal of cyclopropane, it was assumed that the curarized animal was free of the anesthetic during the period from 9 to 15 minutes after shutting off the cyclopropane. It will be noted that statistically there are no significant differences among the averages of R.P. while under the anesthetic alone, with curare alone, and while the animal was both curarized and anesthetized.

The results of this study indicate that the average normal R.P. in fibers of the anterior tibial muscle of the mouse is very close to 100 mV. The average of all measurements is 99.8 mV.; the average of the means of 15 experiments is 99.4 mV.; the smoothed distribution curve peaks very near to 100 mV.; and the most frequently observed value was 101 mV.

DISCUSSION

If the ratio of intracellular to extracellular potassium within a muscle at rest and presumably in a steady state depends solely upon the electro-chemical potential across the cell membrane (Hodgkin, '51), then the R.P. may be expected to correspond to the relative concentrations of potassium ions inside the cell membrane (K_i), and outside the membrane (K_o), according to the equation:

$$E = \frac{RT}{F} \ln \frac{[K_i]}{[K_o]}$$

At 37°C. this equation may be reduced to the form:

$$E = 61.5 \log \frac{[K_i]}{[K_o]}$$

Values for serum and intracellular potassium in the mouse were not found in the literature. Hastings and Eichelberger ('37), however, have listed such values for skeletal muscle of the dog. They determined the serum potassium to be 4.0 mM/

kilo serum water. Intracellular potassium was calculated to be very close to 140 mM/kilo fiber water. Values quoted by Hodgkin ('51) indicate essentially the same levels for cardiac muscle of the rat. Using these values in the above equation the predicted R.P. is 95 mV. Using values determined in this laboratory, namely 142 mM/kilo muscle water in the rat (Humoller, Griswold and McIntyre, '50) and 5.92 mM/kilo serum water for the dog (McIntyre, '32), the predicted R.P. is 85 mV. Obviously the uncertainty concerning the exact ionic concentrations related to a cell in which the R.P. is being measured means that this correlation can be only approximate. However, it may be significant that the mean value for the normal R.P. reported here for the mouse exceeds the value predicted on the basis of known potassium concentrations.

It is also of interest to speculate on the significance of the highest R.P.'s recorded for single fibers. There are at least two possibilities regarding them: (1) those particular fibers have essentially constant potentials higher than the average, or (2) the high R.P. in such a fiber is measured at a time when the voltage is transiently above the mean. The first possibility implies a steady state in which the potential might be expected to be correlated with the ionic distribution on the two sides of the membrane. The second possibility involves no such implication. Assuming the first alternative to be true, it is possible to calculate the ratio of intracellular to extracellular potassium which would be consistent with the highest potential recorded, i.e. 123 mV. In this case the ratio is exactly 100 to 1. Assuming the value of 4.0 mM for serum potassium to be correct, this means that the intracellular potassium concentration would have to be 400 mM. This is obviously impossible when the total intracellular cation concentration is slightly less than 200 mM (Hastings and Eichelberger, '37). Even considering the R.P. as 110 mV. (recall that almost 8% of the recorded potentials were at least that large), the intracellular potassium would have to be greater than 240 mM to establish a ratio consistent with this membrane potential. The discrepancies here pointed out can hardly be accounted for on the basis of possible error in the determination of serum potassium. Therefore, if the movement of potassium across the cell membrane is actually determined by the electrochemical potential (and there is good evidence to support this), one seems forced to the conclusion that the R.P. is a labile voltage, and that high potentials are recorded when abnormally active metabolism (perhaps mediated through a ''sodium pump'') results in a transient hyperpolarization of the membrane.

SUMMARY

1. A semi-automatic micro-electro-forge is described together with the procedure for its use in the relatively consistent preparation of capillary micro-electrodes.

2. Resting potentials of single fibers of the anterior tibial muscle in living mice were measured with intracellular microelectrodes. The data include the results of 1130 determinations in 15 animals, a distribution curve for all recorded values, and an analysis which indicates the mean R.P. to be very close to 100 mV.

LITERATURE CITED

Brock, L. A., J. S. Coombs and J. C. Eccles 1952 The recording of potentials from motoneurones with an intracellular electrode. J. Physiol., *117:* 431–460.

Draper, M. N., and S. Weidman 1951 Cardiac resting and action potentials recorded with an intracellular electrode. J. Physiol., *115:* 74–95.

Dunn, A. L., A. L. Bennett and A. R. McIntyre 1952 Resting potentials of frog ventricle as effected by ouabain and chlorophyll. Proc. Am. Pharmacol. Soc., in press.

Fatt, P., and B. Katz 1951 An analysis of the end plate potential recorded with an intracellular electrode. J. Physiol., *115:* 320–370.

———— 1952 Spontaneous subthreshold activity at motor nerve endings. J. Physiol., *117:* 109–128.

Fingle, E., L. A. Woodbury and H. H. Hecht 1952 Effect of innervation and drugs on direct membrane potentials of embryonic chick myocardium. J. Pharm. and Exp. Therap., *104:* 103–114.

Graham, J., and R. W. Gerard 1946 Membrane potentials and excitation of impaled single muscle fibers. J. Cell. and Comp. Physiol., *28:* 99–117.

HASTINGS, A. B., AND L. EICHELBERGER 1937 The exchange of salt and water between muscle and blood. The effect of an increase in total body water produced by the intravenous injection of isotonic salt solutions. J. Biol. Chem., *117:* 73–93.

HODGKIN, A. L. 1951 The ionic basis for electrical activity in nerve and muscle. Biol. Rev., *26:* 339–410.

HOFFMAN, B. F., AND E. E. SUCKLING 1952 Cellular potentials of intact mammalian hearts. Am. J. Physiol., *170:* 357–362.

HUMOLLER, F. L., B. GRISWOLD AND A. R. MCINTYRE 1950 Effect of neurotomy on the inorganic cation content of skeletal muscle. J. Gen. Physiol., *33:* 723–728.

LING, G., AND R. W. GERARD 1949 Normal membrane potential of frog sartorius fibers. J. Cell. and Comp. Physiol., *34:* 383–397.

MCINTYRE, A. R. 1932 The concentrations and distribution of potassium in the serum and cells in the blood of normal unanesthetized dogs. J. Biol. Chem., *98:* 115–120.

NASTUK, W. L. 1950 Electrical activity of single muscle fibers at the neuromuscular junction. Fed. Proc., *9:* 94.

——— 1951 Membrane potential changes at a single muscle end plate produced by acetylcholine. Fed. Proc., *10:* 96.

NASTUK, W. L., AND A. L. HODGKIN 1950 Electrical activity of single muscle fibers. J. Cell. and Comp. Physiol., *35:* 39–77.

SVAETICHIN, G. 1951 Analysis of action potentials from single spinal ganglion cells. Acta Phys. Scand., *24:* 23 — Suppl. 86.

WARE, F., JR., A. L. BENNETT AND A. R. MCINTYRE 1952a The membrane resting potential in denervated mammalian skeletal muscle measured *in Vivo*. J. Pharm. and Exp. Therap., *106:* 422.

——— 1952b Membrane resting potential of mammalian skeletal muscle measured in vivo. Am. J. Physiol., *171:* 777.

WEIDMAN, S. 1951 Electrical characteristics of sepia axons. J. Physiol., *114:* 372–382.

WOODBURY, J. W. 1952 Direct membrane resting and action potentials from single myelinated nerve fibers. J. Cell. and Comp. Physiol., *39:* 323–339.

WOODBURY, L. A., H. H. HECHT AND A. R. CHRISTOPHERSON 1951 Membrane resting and action potentials of single cardiac muscle fibers of frog ventricle. Am. J. Physiol., *164:* 307–319.

WOODBURY, J. W., AND L. A. WOODBURY 1950 Membrane resting and action potentials from excitable tissues. Fed. Proc., *9:* 139.

WOODBURY, L. A., J. W. WOODBURY AND H. H. HECHT 1950 Membrane resting and action potentials of single cardiac muscle fibers. Circulation, *1:* 264–266.

GLUTATHIONE AND X-RAY INJURY IN HYDRA AND PARAMECIUM

GEORGE E. DANIEL AND HELEN D. PARK

National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service, Federal Security Agency, Bethesda, Md.

In a series of papers Barron and his colleagues ('49) have shown that several of the sulfhydryl enzymes were reversibly inactivated by moderate doses of x rays. Chapman et al. ('50) and Patt et al. ('50) have observed that pre-irradiation administration of glutathione or cysteine affords some protection to mice subjected to an otherwise lethal dose of x ray. In the series of experiments reported here we show a protective action of glutathione, given post-irradiation, when the SH compound is added to the irradiated saline environment of a coelenterate and a protozoan.

EXPERIMENTAL MATERIAL AND RESULTS

The experimental organisms employed were clone cultures of the common brown Hydra and Paramecium caudatum. The hydras were grown in the laboratory in dilute saline (NaCl, .05 gm; KCl, .002 gm; $CaCl_2$, .003 gm; H_2O, 1000 ml) and were fed daily from a culture of Daphnia. The paramecia were cultivated in a water extract of powdered lettuce and supplied *Aerobacter aerogenes* as food. Cultures three to 5 days old were used in the experiments.

All radiations were made with a beryllium window x-ray tube operated at 50 KV constant potential and 50 ma with added filtration as described. Dose determinations were made by the chemical method of Andrews and Shore ('50). This method of dosimetry gives the radiation dose delivered to the solution rather than the air dose as is customary in mammalian experiments. All of the experiments were done in a room maintained at 23° ± 2°C. and during irradiation the vessels containing solution were water cooled to about 20°C.

Glutathione, obtained from the Nutritional Biochemical Corporation, was used, without further purification, in a final concentration of M/100,000 throughout. All water was double distilled, the last distillation being from Pyrex glass and most lots of water were used within 72 hours following the last processing. There was no attempt to prevent casual dust contamination, and the water was in equilibrium with room air.

All irradiation of the animals or the saline used in the Hydra experiments was carried out in a cylindrical dish of approximately 100 ml capacity which contained 50 ml of solution. The dish was loosely covered with an 0.020 in. aluminum sheet.

When the hydras themselves were irradiated the dish as described above was used and the animals were removed to unirradiated saline immediately following treatment. We have shown (Daniel and Park, '51) that irradiated saline is toxic to Hydra but this effect requires some time to develop and we have been unable to observe any ill effects when the Hydra were in contact with irradiated saline for only 10–15 minutes. For this reason we feel sure that results obtained on hydras promptly removed to unirradiated saline represent a response to radiation received by the organisms rather than to a toxic medium.

All of the hydras or saline received one dose of 8,000 r given at a rate of 2,900 r/minute. In all instances the hydras were placed, post-irradiation, in individual cups containing the appropriate solution, maintained in a common moist chamber. Examinations were made every 24 hours. Experiments on Hydra were conducted over a period of several weeks in groups of three to 5 experimental and control animals. In no instance was an experimental group set up without an equal number of controls being taken from the same mass culture.

The criterion of damage by x ray was the degeneration of the tentacles in 24 and 48 hours. In a slightly unfavorable environment the normally highly contractile tentacles of Hydra begin to show signs of damage before any other part of the animal is visibly altered. The 12% of control animals showing damage in 48 hours is due to the inadequacy of the inorganic solution used as the basis of culture medium. When such an inorganic base is modified by the addition of materials arising from the disintegration of regurgitated food residues, secretions of the Hydra themselves, unavoidable carry over of Daphnia culture fluid and from other sources, the tentacles rarely show the condition we have considered as damaged. It is obviously impossible to reproduce such culture conditions so we have accepted as ''normal'' the 10–12% of damaged tentacles arising in the basic inorganic solution. The first indication of damage is usually the appearance of a knob at the distal end of the tentacle. As disorganization proceeds there is a loss of cells, a shortening and thickening of the organ accompanied by a loss of contractility and eventually the complete disappearance of the tentacle. A ''short'' tentacle may be considered as one not longer than the diameter of the hypostome at the point of attachment of the circle of tentacles, thicker than usual, end rounded or roughened, not tapered; less sensitive to mechanical stimulation and incapable

TABLE 1

The effect of direct irradiation and irradiated saline on hydra tentacles

TREATMENT OF		TOTAL NO. OF HYDRA TREATED	PER CENT OF HYDRA WITH DAMAGED TENTACLES	
Hydra	Saline		24 Hours	48 Hours
Unirradiated	Unirradiated	73	1.4	12.3
Unirradiated	Unirradiated + GSH[1]	50	6.0	12.0
Unirradiated	Irradiated	41	78.0	97.6
Unirradiated	Irradiated + GSH[1]	36	0.0	16.9
Irradiated	Unirradiated	36	2.8	63.9
Irradiated	Unirradiated + GSH[1]	36	5.6	44.4

[1] GSH — Glutathione.

of extending to give the tapered appearance of the normal organ. Only those tentacles classified as "short" or less have been considered as degenerated and only those animals having half or more of their tentacles so classified have been considered as being damaged for purposes of calculating the values shown in table 1.

The data obtained from procedures as outlined above are presented in table 1. These data indicate quite clearly that: (1) 8,000 r delivered to the saline is much more effective in producing tentacle damage than is an equal dose delivered directly to the cells of the animal. (2) Glutathione is quite effective in protecting against the damage due to irradiated saline ($P = < .01$) but is ineffective in alleviating or protecting from the damage of direct irradiation ($P = > .10$). (3) The damaging action of irradiated saline is much more evident during the first 24 hours than is that of direct irradiation.

At this point we would like to describe a long series of experiments done on Paramecium in which the criteria of x-ray damage were the number of divisions and deaths occurring in the first 24 hours post-treatment. In these experiments, irradiated water was used in making the environmental media for the experimental groups. Irradiation, using the x-ray tube, and method of dosimetry described above, was carried out in a closed glass flask of rectangular cross section and 200 ml capacity. No filters except the glass of the vessel were used. These conditions permitted an irradiation dose rate of 1,250 r per minute. Following the desired irradiation (in this series total doses of 75,000 r and 150,000 r were given), the water was used to prepare the mixtures of lettuce extract and glutathione. In all cases standard lettuce extract was diluted one to one with irradiated water or plain distilled water. Volume changes due to addition of glutathione to a final concentration of M/100,000 were negligible. In no instance was the lettuce extract, the glutathione or the paramecia irradiated. Single paramecia, taken from a portion of the stock culture which had been diluted with distilled

TABLE 2

Effects of M/100,000 glutathione on Paramecia kept 24 hours in media employing irradiated water (75,000 and 150,000 r) and non-irradiated water as diluent

EXP. NO.	UNIRRADIATED WATER		UNIRRADIATED WATER AND GLUTATHIONE		IRRADIATED WATER		IRRADIATED WATER AND GLUTATHIONE	
	No. div.	No. dead	No. div.	No. dead	No. div.	No. dead	No. div.	No. dead
Eighteen Paramecia in each treatment group of each experiment								
A — 75,000 r								
1	2	3	5	0	5	0	12	1
2	8	0	10	0	5	3	13	0
3	3	2	5	2	1	10	7	1
4	4	..	5	..	3	..	5	..
5	5	0	11	1	1	9	0	1
6	6	0	7	0	1	3	4	2
7	2	1	6	0	1	2	6	0
Total	30	6	49	3	17	27	47	5
B — 150,000 r								
8	4	1	8	2	1	3	6	1
9	5	0	11	0	0	7	5	2
Total	9	1	19	2	1	10	11	3

water to the same lettuce concentration as that of the experimental and control series were isolated in the depressions of a clear Pyrex glass spot plate containing approximately 0.75 ml of experimental or control solution. The results of these experiments are presented in table 2, A and B, from which it can be seen that there is a marked protection offered by the glutathione.

DISCUSSION

The experiments on a Paramecium of the caudatum type indicate that there is produced in water by x-ray irradiation some substance or substances which prevent the division of the cells. This action is counteracted by the presence of M/100,000 glutathione in the cell environment. Not only is the deleterious effect of the irradiation counteracted but a stimulating effect is observed which is equal to that produced by glutathione in unirradiated media.

Voegtlin and Chalkley ('30) showed that in *Amoeba proteus* the effect of glutathione on the growth of the nucleus and the division of the cell was correlated with the size and therefore with the physiological age of the cell. Back and Halberstaedter ('45) demonstrated that the effect of x rays on Paramecium was greatly affected by the history of the culture immediately preceding the exposure to x rays. In the experiments reported in the present paper we were not concerned with determining the minutia of the physiological relationships in the cell-glutathione-x-ray complex but were interested in determining whether or not a sulfhydryl containing compound, when added to the environment of a cell or tissue, would exert any protective action against x-ray irradiation damage in a population which had only a reasonable degree of homogeneity in the physiological ages and conditions represented.

Irradiated saline produces 78% damage to Hydra tentacles in 24 hours and 97% in 48 hours. It is known from some of our unpublished experiments that irradiated saline retains a high degree of toxicity for at least 5 days. Therefore, the animals are subjected to continuing action of the unfavorable environment. Such a solution apparently readily affects the cells and damage occurs quite rapidly. The variation in physiological stamina of the cell population would explain the increase from 78% to 97% in the second 24 hours of observation.

There is no certain indication that the active substance produced in the cell by direct irradiation is the same as that formed in the irradiated saline although the minimum basic ingredients, water, oxygen and salts, are present in both cases. If we assume that damage from irradiated saline and direct irradiation of the cell is due to the same chemical compounds the difference in time of appearance of damage could be explained on the basis of diffusion. The concentration of products in irradiated saline is probably higher (because of greater solubility of oxygen and other gases in saline) than could be produced by equal doses of x rays from intracellular water, oxygen and salts. Some of the products are readily diffusible and the equilibrium concentration between cell and fluid may be high enough to rapidly disrupt the physiology of the cell.

When cells are irradiated and rapidly removed to unirradiated fluid the diffusion of toxic material is from the cell to the outer environment with a consequent lowering of intracellular concentration of toxic material. Diffusion, plus the reaction of cellular components with the toxic material keeps the concentration low enough so that damage is not immediately apparent. Impairment of cell function has occurred, however, and the metabolic disturbance is manifest after a longer period than is observed in the case where a larger dose of toxic material is administered via irradiated saline.

The mechanisms outlined above are plausible on the assumption that the toxic material is the same in both irradiated saline and irradiated cell. There can be little doubt concerning peroxide production in the cell but just as certainly there is an opportunity for many harmful substances other than peroxides to be produced. While the effect of x rays on amino acids or proteins is not too clearly understood there is no question as to the actuality of an effect. Alteration in the structural configuration of a protein forming part of the semi-permeable plasma membrane may well lead to the disintegration of the cell and consequent organic degradation.

The addition of glutathione to irradiated saline and the subsequent introduction of unirradiated cells is essentially an example of simultaneous treatment. Assuming that part of the damage is due to H_2O_2 it is reasonable to suppose that the glutathione will be oxidized to the disulphide thus reducing the concentration of the peroxide and at the same time leaving the glutathione available for metabolism. (See Voegtlin and Chalkley, '31, for utilization of SH and SS.) The reduction of peroxide concentration would probably proceed faster than the apparent manifestations of damage thus giving a picture of protection in the glutathione solutions. The events observed would thus be the result of a purely chemical reaction taking place in the solution and in no way involve the physiological activity of reduced or oxidized glutathione.

SUMMARY AND CONCLUSIONS

1. 8,000 r of x ray produces in water or saline substances which damage the tentacles of Hydra. The damaging action is marked after 24 hours and increases to about 100% of the animals in 48 hours.

2. When unirradiated Hydra are put into irradiated saline containing M/100,000 glutathione the damaging effects of the solution are not observed.

3. 8,000 r of x rays produces in saline toxic substances which have a greater damaging effect on Hydra tentacles in 24 hours, than is produced by an equal dose delivered directly to the cells of the animal.

4. Glutathione apparently has no effect on the number of paramecia dying in unirradiated solutions. Irradiation of the medium produces a considerable increase in the number of deaths and this action of irradiation is offset completely by the glutathione. Our data show that there is no difference between the killing action of H_2O receiving 75,000 and 150,000 r.

5. X-ray irradiation produces in water substances which inhibit the division of Paramecium. This inhibition is prevented by glutathione.

6. A suggestion is made concerning the mode of action of glutathione in preventing the deleterious action of irradiated media.

LITERATURE CITED

ANDREWS, HOWARD L., AND PARKHURST A. SHORE 1950 X-ray dose determinations with chloral hydrate. J. Chem. Physics., *18:* 9, 1165.

BARRON, E.S.G., S. R. DICKMAN, J. A. MUNTZ AND T. P. SINGER 1949 Studies on mechanism of action of ionizing radiations. I. Inhibition of enzymes by X-rays. J. Gen. Physiol., *32:* 537–552.

CHAPMAN, W. H. 1950 Sulfhydryl containing agents and the effects of ionizing radiation. I. Beneficial effects of Glutathione injection on X-ray induced mortality rates and weight loss in mice. Radiology, *55:* 6, 865–873.

DANIEL, GEORGE E., AND HELEN D. PARK 1951 The effect of X-ray treated media on Hydra tentacles. J. Cell. and Comp. Physiol., *38:* 3, 417–425.

PATT, H. M. 1950 Further studies on the modification of sensitivity to X-rays by cysteine. Proc. Soc. Exp. Biol. and Med., *73:* 1–18.

VOEGTLIN, CARL, AND H. W. CHALKLEY 1931 The chemistry of cell division. I. The effect of glutathione on cell division in Amoeba proteus. Public Health Reports, *45:* 50, 3041.

THE GENERAL FORM OF CIRCULATION IN THE LOBSTER, HOMARUS[1]

J. WENDELL BURGER AND CHEVES McC. SMYTHE[2]
The Mt. Desert Island Biological Laboratory, Salisbury Cove, Maine; Trinity College, Hartford, Conn.; and the College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York, N. Y.

THIRTEEN FIGURES

While an extensive literature exists on many phases of the circulatory physiology of the higher Crustacea, little attention has been given to the development of an integrated picture of circulatory mechanics for the system as a whole. The circulation is a low pressure type (cf. Prosser et al., '50, for a summary) but it is not clear if the system is primarily a heart driven system or one in which a pool of hemocoelar blood is moved about by muscular activity that is largely non-cardiac. We have tried to assemble a set of elementary data for the lobster, and to a lesser extent, the crab. which permits a generalized coherent picture. It was possible to use sensitive modern recording equipment, and to employ techniques which made unnecessary the opening of the carapace which destroys the integrity of the circulatory system. In dealing with these data, the peculiar circulatory anatomy of the lobster must be understood. Herrick ('09) has partially described the system for the lobster, but a more informative account is that of Pearson ('08), although for the crab.

---

[1] Aided by grants from the American Heart Association and the National Heart Institute, U. S. Public Health Service.

[2] Fellow of Life Insurance Medical Research Fund.

METHODS

Lobsters *(Homarus americanus)* secured from a commercial pound in the same bay as the Laboratory were kept in live cars and fed freely on fish. Since lobsters rapidly lose water in air (Cole and Kazalski, '39), care was taken to use only fresh, always submerged animals. For laboratory study, the lobsters were tied in running sea water (16°–20° C.) to the narrow edge of a piece of a weighted 2″ × 4″ plank by a cord across the uropods and telson and another across the anteriorly extended chelae, avoiding pressure on the joints of the chelae. In this position, the periopods were free and respiration unhindered.

Blood pressure measurements were taken by a strain-gauge recording through a string galvanometer. An 18 gauge needle was attached to lead tubing running to the gauge by a firm rubber collar, since it was desirable to have a little play during cannulation. Once the needle was in position, the tubing was refilled. By inserting the needle in the dorsal abdominal surface between the second and third segments, one can blindly cannulate the interior of the ventricle, the cardiad end of the posterior abdominal artery, the pericardium, and adjacent sinuses of the hemocoel. Dissections of the needle were made until a certain correlation between the position of the needle tip and various pressure curves was established. Cannulations of various parts of the hemocoel were made externally, although for the branchio-pericardial "veins," it was found desirable to cut off a piece of the gill-cover to insure accurate cannulation. Here blood loss was small since no large tissue mass or blood space was cut. *Cancer irroratus* was also explored, although blind cannulations are more difficult to make. The advantage of this technique is that *intact* animals are being studied, and can be used over and over again. In the data presented here, the animals were not used over again; each animal mentioned is a fresh, previously untreated lobster. Other techniques employed are mentioned below in context with their use.

RESULTS AND COMMENT

Intra-ventricular blood pressure[3] for 14 "resting" intact lobsters (*c* 425–500 gm), at 16°–20°C., for heart rates of 50–136 per minute, gave a systolic range of 9–22 mm Hg, and a diastolic range of 0–5 mm Hg. The most frequent systolic pressure was 12 ± 1 mm Hg (6 of 14 lobsters); two animals had pressures just under 10 mm Hg and 6 had systolic pressures over 15 mm Hg. The most frequent diastolic pressure was 1 mm Hg (7 animals); two were approximately zero, and 5 were 3–5 mm Hg. Pulse pressures ranged between 8–16 mm Hg, most frequently 12 ± 2 (8 animals). The above data indicate a generalized normal intra-ventricular blood pressure of approximately 13/1 mm Hg. Intra-ventricular pressure curves are shown in figures 1–4.

In the quiet animal, the pulse pressure did not vary with either the systolic or diastolic pressure, e.g., a low systolic or diastolic pressure was not correlated with a low pulse pressure. No correlation was found between intra-ventricular pressure and heart rate over a range of 50–136 beats per minute (cf. figs. 1–3), nor between ventricular pressure and body size (cf. figs. 1–2). This latter statement is based on a comparison between the above animals and three large lobsters *c* 1080, 1470, 1720 gm.

Immediately behind the ventricle is an arterial chamber from which the posterior abdominal artery and the ventrally directed sternal artery arise. The systolic pressure in this chamber (artery) was essentially that of the ventricle, while the diastolic pressure was 3–5 mm Hg less (cf. figs. 2, 3). Our data indicate a correlation between arterial diastolic pressure and heart rate; the diastolic pressure falling more with lower heart rates. The generalized mean arterial pressure immediately outside the ventricle can be considered as approximately 11 mm Hg. The pulse and mean arterial pres-

---

[3] The zero reference plane was set at the level of the heart. Theoretically and practically a correction factor was indicated. The magnitude of this factor could not be determined exactly, but did not exceed 2 mm Hg. Hence, an arbitrary 1.5 mm Hg was added to the values secured. This addition should be borne in mind.

sure undoubtedly decrease distally. Brücke and Satake ('12) found for the lobster, a mean arterial pressure of 4–14 mm Hg in the posterior abdominal artery at the third and 4th segments. Realizing that their manometer was damped they suggest a probable pulse pressure of 1.5 mm Hg.

Since the arteries are thin delicate transparent tubes, it does not seem that vasomotive changes or factors of arterial elasticity play important hemodynamic roles, although this matter was not studied directly. The arteries are immediately surrounded by hemocoelar blood which almost certainly must resist the systolic thrust, and impart a circumambient elasticity. Perhaps the compressive effect of the hemocoelar blood permits the lobster to have such delicate arteries.

With muscular movements or with artificial compression of the carapace, intra-ventricular pressures rose (cf. fig. 10). One unrestrained lobster actively moving on the bottom of a tank showed a changing variety of pressures, the maximum being 27/13 mm Hg. These pressure rises are due to increased venous pressure (cf. below). While the heighth of the pulse curve tended to fall with larger pressures, the pulse curve was never abolished, even with pressures above 50 mm Hg. It is important to note that the systolic pressure was always higher than pressures circumambient to the ventricle. The heart is strapped dorsally and laterally (not ventrally) to the pericardial wall by numerous ligaments (alae cordis with alary muscles) which pull the contracted ventricle back to an expanded state. This arrangement undoubtedly permits the ventricle to fill despite surrounding positive pressures. If these ligaments are cut, the heart contracts into a twisted mass, which no longer fills with blood.

In our intact quiet animals, variations in heart rate and in pulse heighth were inconspicuous. Sharp pressure rises were sometimes associated with a transitory bradycardia, and a fall in pulse heighth. Smith ('47) with perfusion of the heart of *Cancer* found that a decreased perfusion rate was followed by decreased heart rate and amplitude. It is probable that the above slowing was due to decreased ventricular filling resulting from the compressive action of the increased pressure. Spontaneous bradycardia was noted a few times in resting lobsters. Draining away water from the animals was associated with a fall in blood pressure and heart rate, both of which returned to previous levels on resubmergence. In general under our experimental conditions, evidence was not found for a continuous interplay of cardiac adjustments. This does not mean that these mechanisms are ineffectual and inoperative.

Other reports of intra-ventricular pressures are: *Maja,* mean pressure *c* 4 mm Hg (Dubuisson, '28); *Cambarus,* average *c* 6.4/5.9 mm Hg (Inada, '47). In the crayfish *Cambarus,* the systolic pressure is roughly half that of the lobster, the pulse pressure was recorded as only about 4% of that of the lobster. This raises the question as to whether Inada's measuring of pressures with an exposed pericardium gave natural results.

After flowing through the post-arterial "capillaries" (arterial terminations), the blood enters the sinusoidal hemocoel. Despite a bewildering array of channels, sinuses, membranes, baffles, etc., India ink injected under pressure from a 2 ml syringe into a fresh animal with its heart removed, was recovered from all portions of the hemocoel except the transbranchial portion (pericardium and branchio-pericardial "veins"), including such fine portions as the antennae and swimmerets. This indicates that the hemocoel is a unit sinus; blood and pressure can be transmitted from one point throughout the whole hemocoel. This leads to the impression that the hemocoelar blood may move erratically about like water agitated in a tank.

Examination of the living animal indicates that this impression is erroneous. If dye is gently introduced into the hemocoel where it can be followed visually such as under the ventral surface of the abdomen, between the joints of the legs and under the gill covers, the dye in a quiet animal flows *unidirectionally* toward the gills in thin vein-like streams. In the tail, two thin streams flow anteriorly; in the periopods the flow is also in two thin streams, which when they leave the legs flow principally to the gills of that leg. On flexion of the abdomen the dye was driven forward toward the gills. If the tail or legs are compressed, the dye did squirt in all directions, but on release of the compression, it again resumed a unidirectional flow. While the whole hemocoel could not be studied visually, our observations indicated clearly that where flow was visible it was unidirectional, and flowed in streams comparable to veins. In the intact animal the tissues are so disposed as to block out vein-like channels which have no real walls. Under pressure blood can push out of these channels in all directions. In the Vertebrate with closed veins blood under applied pressure can only distend the veins laterally and move forwards and backwards. Our observations in the lobster do not support the commonly held idea that the blood in the hemocoel is erratically pushed about by muscular movements of an extra-cardiac nature. Like the Vertebrate the lobster appears to have an *orderly* circulation which is influenced by activity of skeletal muscles.

The elementary mechanism of this orderly flow seems clear. The systolic pressure is superior to the hemocoelar pressure (cf. below), so that over the whole animal the hemocoelar blood is pushed by a superior arterial pressure. The gills and transbranchial circulation furnish the only outlet for the hemocoelar blood which flows therefore in the direction of the gills.

Linear blood flow as measured by the flow of dye in the ventral abdominal sinus and in the periopods was about 0.3–0.6 cm per second for a quiet lobster. On flexion of the abdomen, flow in the ventral abdominal sinus increased up to 5 cm per second. Stagnation of dye for short periods was observed, and also imperfect unidirectional flow.

Pressures [4] in the semocoel of a quiet lobster, before cardiac cannulation, as measured in the sternal sinus, dorsal and ventral abdominal sinuses, and sinuses of the chelae and periopods and at the base of the antennae were about 2–6 mm Hg, and uniform over the whole animal (cf. figs. 5–9). Faint traces of an arterial pulse were visible in some records, and could be seen sometimes as oscillations in injected dye. On movement of the legs, etc., the hemocoelar pressure rose (cf. fig. 10), but returned to resting levels on cessation of movements. Of course, small local pressures are not transmitted fully over the whole animal, as the lobster is not completely rigid. Prosser, Inada and Zuckerkandl ('47) for *Cambarus* found persistent differences in various parts of the hemocoel, with the distal chelae having pressures higher than cardiac pressures. If this latter situation is a permanent one it is hard to see how the arteries can deliver blood to the chelae. The small size of the crayfish makes pressure measurements more hazardous than for the larger lobster. It should also be noted that Crustacea make many small limb movements and frequently keep the limbs in tonic contraction. With so many appendages, it is difficult to distinguish various states of activity. The hemocoelar blood pressure is elevated on movement, but we could not study free lobsters with multiple simultaneous measurements.

---

[4] See footnote 3, p. 371.

Other reports for hemocoelar pressures are: *Maja* inactive, base of 4th leg, 2.0–2.5 mm Hg; little variation in one animal, but rising and oscillating irregularly on movement (Dubuisson, '28); *Potamobius* averaged 7.3 mm Hg with a range of 5.5–18.7; *Carcinus* averaged 9.6 mm Hg with a range of 3.6–19 (Picken, '36); *Cambarus,* a range of 5.9–15.4 mm Hg (Inada, '47). Our findings agree most closely with Dubuisson.

The ''venous'' hemocoelar blood must return to the heart by way of the gills; injections of India ink determined that there are no by-passes. The afferent branchial veins control the blood flow through the gills, and protect the pericardium from overloading probably, simply through their diameters. The total filament volume for a *c* 450 gm lobster is about 10 cm^3. The afferent pressure outside the gill is that of the hemocoel. The efferent channels, branchio-pericardial sinuses or ''veins'' are without valves. They and the pericardial cavity have the lowest pressures of the circulatory system (cf. figs. 2a, 11). In the quiet lobster pressures here are close to zero[5] with a pulse variation of about 1 mm Hg. As in other parts of the system, pressure rose here on muscular movement, artificial compression of the body, or compression of one or more gills. The pericardial dynamics are quite complex. We confirm Dubuisson ('28) that dorsal to the ventricle the pressure rises slightly on systole but falls laterally and ventrally. Since ostia are located dorsally, since blood flows into the pericardium when it is open to air, and since the thin pliable pericardial membrane is surrounded by a positive hemocoelar pressure, it appears that postbranchial circulation is not due to a cardiac aspiration.

Blood flow through the gills is doubtlessly aided by the negative pressure of the sea water around the gills but under the gill covers (cf. fig. 12). Water is drawn into the branchial chambers by the gill-bailers which may beat at over 200 beats per minute. In the chambers, the hydrostatic pressure is always negative to the water outside, —1 to —3 mm Hg with an oscillation of 1–2 mm Hg. This circumambient negativity is not essential since lobsters can live for long periods in cold moist air. In air, however, the cardiac rate and pressure fall, probably indicating a more sluggish circulation.

The cardiac output of intact lobsters was crudely determined as 0.1–0.3 ml per beat, which gives minute volumes at the rather usual heart rate of 100 per minute of 10–30 ml. An untapered glass tube, 2 mm inside diameter with jagged edges was blindly plunged horizontally into the posterior ventriculo-arterial aperture. The amount of blood pumped into the open tube per beat was measured. Needless to say, this technique works only if it succeeds on the first thrust, and is not recommended with a limited supply of animals.

The Evans Blue space (T1824) was determined spectrophotometrically. It was found essential that the spectrophotometer be calibrated against the blood taken before injection of the dye. Calibrations for one animal could not be used for another. It was also found desirable to dilute the blood with saline, such as one to 20, rapidly stirring the saline as the blood was introduced. In this way, a uniformly clear unclotted solution can be obtained. All dye dilutions, etc., were made of the same saline solution, 2% sodium chloride buffered a bit above neutrality by bicarbonate. Without the above precautions widely varying results were obtained.

---

[5] See footnote 3, p. 371.

For 5 lobsters the following Evans Blue space was found: 66 cm^3 (14 oz. animal); 72 cm^3 (14 oz.); 72 cm^3 (16 oz.); 76 cm^3 (16 oz.), and 75 cm^3 (17 oz.). The curves could be drawn to yield volumes up to 16% higher. The maximum amount of blood that could be drained and squeezed out of a rapidly macerated animal was 35 cm^3. Taking 75 cm^3 as the T 1824 space of a pound lobster, the ratio of the dye space to body weight is roughly 0.17, and the ratio of dye space to body volume (380 cm^3) is 0.2. Recently Prosser and Weinstein ('50) reported that the mean volume per cent of body weight of *Cambarus* to average 25.1% for the T 1824 space, and 25.6% for the sodium thiocyanate space. The ranges were quite large: 9.9–42.8% for the T 1824 space, and 16.6–47.6% for the thiocyanate space. Drawing our curves to yield maximum volume gave a ratio of T 1824 space to body weight of only 0.19. Samples drawn from various parts of the lobster showed a prompt mixing of the dye. It should be noted that a lobster looks deceptively large due to the gill-covers, lateral extensions of the abdominal carapace, uropods, and internal leg skeleton.

With a minute volume of about 10–30 ml, and a T 1824 space of 75 ml, it would appear that the lobster can turn over its blood volume in 3–8 minutes, an estimate entirely consonant with comparisons of blood drawn from the hemocoel and the pericardium after injection of Evans Blue and phenol red. The circulation is visibly moderately rapid.

Since the carapace appears fairly rigid, it might be supposed that the hemocoelar space has a constant volume. Actually two organs offer room for a very considerable volume adjustment, viz., the nephridial bladders and the stomach. The nephridial bladders are usually distended with urine and contain up to 6 ml for a 450 gm animal. The volume of the stomach was not measured but definitely is greater than that of the bladders. In connection with a study of dye excretion, it was found that many lobsters with otherwise empty stomachs, have stomach distended with sea water. This water was not swallowed in fright as the animals were plucked quickly from shallow tanks. The role, if any, of this swallowing of water was not determined, but the drinking of water by fishes is brought to mind. These two organs, however, do offer a possible mechanism for balancing blood volume within a relatively rigid carapace.

Some pharmacologic studies were made on the intact lobster. Acetylcholine applied peri-ventricularly gave results like those described by Welsh ('42) for the isolated heart: an acceleration blocked by atropine, but with systolic stoppage with large doses. When this drug was applied intra-ventricularly it had no action, indicating that it acts on the cardiac ganglia and not on the ventricular muscle directly. Racemic epinephrine applied both around and within the ventricle had no effect on rate and blood pressure except with massive doses where the rate, pulse, and pressure increasingly became reduced and ultimately obliterated. Similar results were secured by Brücke and Satake ('12) and Cole and Kazalski ('39). Nicotine initially increased heart rate and blood pressure, followed by cardiac depression and fall in pressure.

Six crabs, *Cancer irroratus*, *c* 275 gm were partially explored. The cardiac pulse and pressure relations were the same as for the lobster, except that the heart rate was faster, *c* 150 beats per minute, and the intra-cardiac pulse was lower, *c* 8 mm Hg (cf. fig. 13).

SUMMARY

In order to develop a coherent picture of the whole circulation in the Decapod Crustacea, a set of elementary data was secured on the *intact* lobster, *Homarus americanus*. Blood pressure measurements were taken by a strain-gauge activating a string galvanometer. The following information resulted:

1. In the quiet lobster, intra-ventricular blood pressure was most commonly 13/1 mm Hg (systolic range 9–22; diastolic range 0–5 mm Hg). No correlation between heart rate or body size and cardiac blood pressure was found. Aortic blood pressure immediately posterior to the ventricle was the same as in the heart for systole, and 3–5 mm Hg less on diastole. The diastolic aortic pressure was correlated with the heart rate. Cardiac and aortic blood pressures, together with pressures in all parts of the circulatory system rose on body movement or on artificial compression of the carapace. Despite large circumambient pressure rises, to over 50 mm Hg, the cardiac pulse was not obliterated.

2. In the quiet lobster, hemocoelar blood pressure was about 2–6 mm Hg, and uniform over the whole animal. Pressure in the branchio-pericardial sinuses and in the pericardium were the lowest of the whole system, being about zero with an oscillation of about 1 mm Hg. Pressure changes in the pericardium are not uniform for all parts of the pericardium.

3. Injection with India ink determined that the whole hemocoel except the transbranchial portion can be considered as a unit space. Observation of the flow of dye where visible through the carapace showed that in the intact animal at rest, the blood flows in the hemocoel *unidirectionally* toward the gills in thin vein-like streams at 0.3–0.6 cm per second. While the dyed blood may be arrested and pushed backwards on movement or the application of local pressure, its tendency is clearly to flow towards the gills. No evidence of the cardiac aspiration of blood was found. Sea water around the gills is always at an oscillating negative pressure.

4. The cardiac stroke volume was crudely determined as 0.1–0.3 ml. The T 1824 space of lobsters *c* 450 gm was approximately 75 cm^3. The ratio of this space to body weight is approximately 0.17, and to body volume approximately 0.2. The circulation is visibly, moderately rapid. The above data together with a rather common heart rate of 100 beats per minute, indicate that the lobster can turn over its blood

volume in 3–8 minutes. It is suggested that the nephridial bladders and the stomach provide a mechanism for adjusting blood volume within a fairly rigid carapace.

5. Pharmacologic studies with acetylcholine, epinephrine, and nicotine on the intact animal gave no new information. Acetylcholine applied intra-ventricularly had no effect, indicating that this drug acts on the cardiac ganglia and not on the ventricular muscle directly. In our preparations, cardiac rhythm was quite steady in any one animal, and we did not observe the interplay of adjustment mechanisms.

6. Partial exploration of *Cancer irroratus* showed cardiac pulse and pressure relations the same as the lobster, except that the heart rate was faster, *c* 150 per minute, and the intra-cardiac pulse was lower, *c* 8 mm Hg.

7. It is concluded that in the intact lobster the circulation is primarily heart driven. The hemocoelar blood flow is markedly unidirectional. Some of this blood at least, flows in vein-like streams. We cannot support the widely held view that the blood is erratically propelled about the hemocoel by non-cardiac forces. On muscular movement, the pressure of the whole system is raised, but the systolic pressure still is the superior pressure. As in the Vertebrate, body movements and contraction of skeletal muscles furnish an adjunct to a heart driven circulation.

LITERATURE CITED

Brücke, E. T., and J. Satake 1912 Der arterielle Blutdruck des Hummers. Zeitschr. alleg. Physiol., *14:* 28–38.

Cole, W. H., and L. A. Kazalski 1939 A perfusing solution for the lobster heart and the effects of its constituent ions on the heart. Bull. Mt. Desert Isl. Biol. Lab., 40–46.

Dubuisson, M. 1928 Recherche sur la circulation du sang chez les Crustacés 2^{e} note: Pressions sanguines chez les Décapodes Brachyures. Arch. de Biol., *38:* 9–21.

Herrick, F. H. 1909 Natural History of the American Lobster. Bull. Bureau of Fisheries, U. S. Dept. Com. and Lab., *29:* 153–384.

Inada, C. S. 1947 Comparison of the blood pressures in open and closed types of circulatory system. Master's Thesis, Graduate School, the Univ. of Illinois.

Pearson, J. 1908 Liverpool Marine Biol. Com. Memoirs. XVI. Cancer. London.

Picken, L. E. R. 1936 The mechanism of urine formation in invertebrates. I. The excretion mechanism in certain Arthropoda. J. Exp. Biol., *13:* 309–328.

Prosser, C. Ladd et al. 1950 Comparative Animal Physiology. Philadelphia, Pa.

Prosser, C. Ladd, C. Inada and E. Zuckerkandl 1947 Pressure regulation in animals with ''open circulation.'' Anat. Rec., *90:* 601.

Prosser, C. Ladd, and S. J. F. Weinstein 1950 Comparison of blood volume in animals with open and closed circulatory systems. Physiol. Zool., *23:* 113–124.

Smith, R. I. 1947 The action of electrical stimulation and of certain drugs on cardiac nerves of the crab, Cancer irroratus. Biol. Bull., *93:* 72–88.

Welsh, J. H. 1942 Chemical mediation in Crustaceans. IV. The action of acetylcholine on isolated hearts of Homarus and Carcinides. J. Cell. and Comp. Physiol., *19:* 271–279.

PLATE 1

EXPLANATION OF FIGURES [6]

b.l. = base line, ar. = artefact. Horizontally, each cm = 0.8 sec.; vertically, each mm = 3 mm Hg, except for figure 11 where 1 mm = 1.7 mm Hg.

1 a, Intra-ventricular pressure curve in quiet intact lobster; b, the same after bending of chela. The sharp thrusts at the beginning of systole and diastole are overshoots of the string. Animal *c* 460 gm. In b, note regularity of heart.

2 a, Aortic pressure curve immediate posterior to heart; b, intra-ventricular pressure curve of same lobster. Animal *c* 1720 gm. Note pressure is not greater than in smaller animal (figure 1).

3 a, Aortic curve; b, ventricular curve at slower heart rates than in figures 1, 2. Note larger aortic pulse than in figure 2. Animal *c* 460 gm.

4 a, Lateral pericardium; b, ventricular pressure curves.

5–9 Hemocoelar pressures in a quiet single animal: 5, dorsal abdominal sinus; 6, sinus at base of antenna; 7, base of chela; 8, base of third periopod; 9, base of 4th periopod. Pressure was uniform over the animal.

10 Pressure curve in sternal sinus with an active waving of the legs. Pressure elevated over resting level, but falling with a return to resting level. The level before movement was the same as shown near the base line at the right end of the strip.

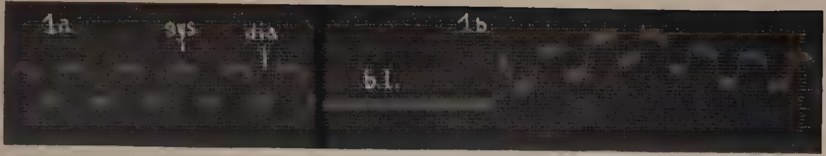
11 a, Middle branchio-pericardial sinus (carrying blood from gills to heart) in a quiet animal; b, with movement of legs. Form of curve in a like that in pericardium (fig. 4 a). With movement, pressure elevated as in other parts of system.

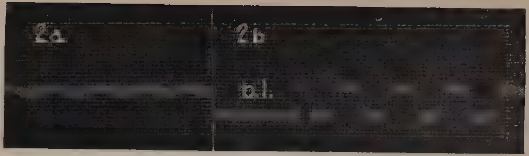
12 Pressure of sea water in branchial chamber under gill covers. Rhythmicity due to gill bailers. For this figure the needle was thrust along the side of the gill bailer from the front. More impressive curves were secured by pushing the needle through the gill cover from the side; in this way, there was no interference with the movement of the gill bailers.

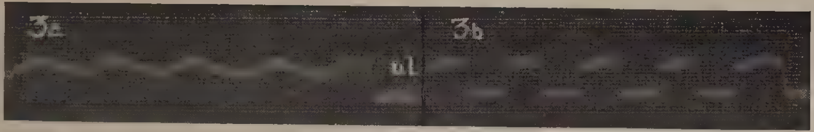
13 Intra-ventricular pressure curve from *Cancer irroratus*. Curve similar to that of lobster, but with smaller pulse. Heart rate more rapid.

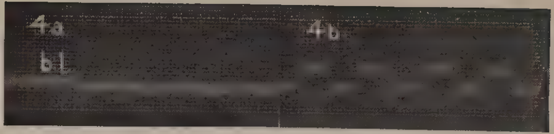
---

[6] The zero reference plane was set at the level of the heart. Theoretically and practically a correction factor was indicated. The magnitude of this factor could not be determined exactly, but did not exceed 2 mm Hg. Hence, an arbitrary 1.5 mm Hg was added to the values secured. This addition should be borne in mind.

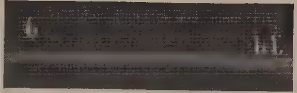

1a
sys
dia
1b
b.l.

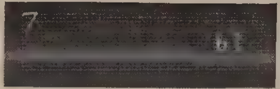

2a
2b
b.l.


3a
ul
3b

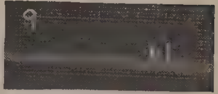

4a
bl
4b

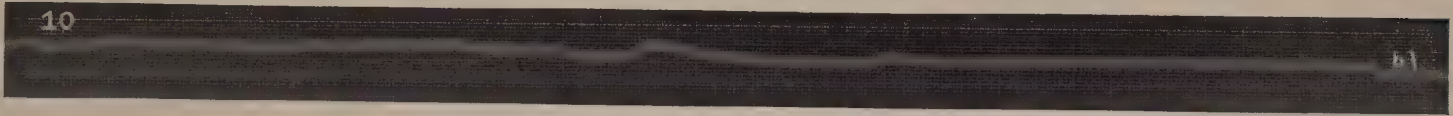
5
bl

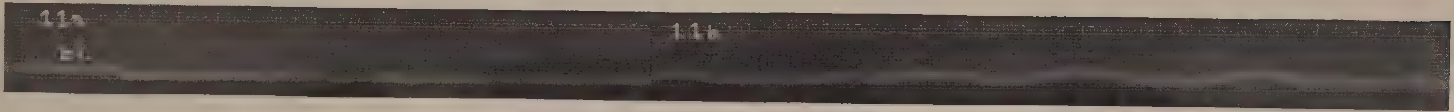

6
bl

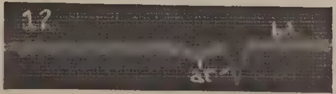

7
bl

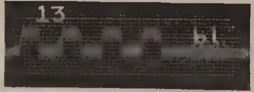
8
bl


9
bl


10
bl


11a
bl
11b


12
bl
ar


13
bl

TRANSPORT OF PHENOL RED IN THE FLOUNDER RENAL TUBULE[1]

KARLMAN WASSERMAN, E. LOVELL BECKER AND
ALFRED P. FISHMAN[2]
*Department of Physiology, Tulane University, New Orleans, Louisiana,
Department of Physiology, New York University College of Medicine,
New York and the Mount Desert Island Biological Laboratory,
Salisbury Cove, Maine*

ONE FIGURE

This report is concerned with the basic mechanisms involved in the renal tubular transport of phenol red, as observed in *in vitro* preparations of the flounder kidney. The procedure is a modification of that developed by Forster ('48) and permits multiple, sequential microscopic observations on the transport of the dye from the medium across the cell and into the lumen.

The particular factors explored were: (1) self-blockade as induced by concentrations of phenol red; and (2) the influence of a carbonic anhydrase inhibitor (Diamox or 6063) on acid secretion by the tubule. These observations bear on the blockade of tubular transport of *p*-aminohippuric acid (PAH) and diodrast at high plasma concentrations in mammals, as revealed by self-depression of Tm and reported by Eggleton and Habib ('50), Barclay, Cooke and DeMuralt ('49), and Schachter and Freinkel ('51), and particularly on the intracellular accumulation of diodrast in the rabbit kidney during overload, as reported by Josephson and his co-workers ('49, '50, '53).

---

[1] This investigation was supported in part by a research grant (USPHS H-1172 [C]) from The National Heart Institute, of the National Institutes of Health, Public Health Service and in part by a grant from the New York Heart Association.

[2] Established Investigator, American Heart Association.

METHODS

Freshly caught winter flounder, *Pseudopleuronectes americanus,* were used. Teased fragments of the kidney (less than 5 mg) were immersed in two-inch petri dishes containing 5 ml of medium. The basic medium, except where otherwise indicated, contained the following salts (mM/L): NaCl, 134; KCl, 2.5; $CaCl_2$, 1.5; $MgCl_2$, 1.0; NaH_2PO_4, 0.5; $NaHCO_3$, 40; phenol red, 0.056 (2 mg). The phenol red concentration, which is higher than that used by Forster and Taggart ('50) and Puck, Wasserman and Fishman ('52), proved more satisfactory in our hands in that dye could be observed to accumulate in a larger proportion of tubules. The $NaHCO_3$ concentration, also higher than previously used, was necessary to maintain an alkaline reaction in the urine at phenol red concentrations above 30 mg %. Oxygen was bubbled through the media and the temperature was kept between 25 and 28°C.

In most instances, the concentration of phenol red in the tubule cells and tubular urine was estimated by visual inspection under the microscope and assessed according to an arbitrary numerical scale. In some instances, however, the concentration was measured by the quantitative comparator block described by Puck, Wasserman and Fishman ('52), except that concentrations were expressed in terms of the equivalent filter concentration rather than as absolute concentration of dye in the tubular urine, since the intrinsic features of the method preclude the exact measurement of the latter. Most observations were made at a magnification of 125 × and with filters having a depth of 0.32 mm.

Occasional tubules did not behave like the rest of the preparation, and in such instances the "physiological condition" of the preparation as a whole was evaluated in accordance with the behavior of the majority of tubules. Observations were made at 20 and 40 minutes after the start of the experiment, because at this time the concentration in the tubular urine had a maximal value and deterioration of the preparation had not yet begun.

Where tubules were immersed in a medium containing more than 2 mg % of phenol red, it was necessary to transfer them to dye-free medium before making quantitative observations.

RESULTS

Effects of calcium and potassium on phenol red transport. Josephson ('49, '50) and Puck et al. ('52) have postulated that at least two steps are involved in the tubular transport of diodrast and phenol red, respectively. Puck et al., in their studies of phenol red, have shown that the first step involves an active uptake of the dye from the medium, and for this reaction potassium is necessary in the medium, the rate of uptake increasing with increasing potassium concentration. The second step is represented by the excretion of the dye into the tubular urine, and requires calcium; in a calcium-free medium the dye accumulates in the cells without significant deposition in the tubular urine, the extent of intracellular accumulation being proportional to the potassium concentration. We have confirmed both of these observations. At a potassium concentration of 1.2 mM/L, tubular transport works effectively to establish a high concentration in the lumen with no accumulation in the cell. But as the potassium concentration is increased, transport to the lumen is depressed and the dye begins to accumulate in the cell. The concentration of potassium required to depress excretion into the urine and to cause accumulation in the cell varies from experiment to experiment. Whether the accumulation of dye in the cell at elevated potassium concentration is attributable to specific blockade of the mechanism that moves the dye from cell to lumen, or acceleration of the reaction by which the dye is taken into the cell to such a degree as to block tubular transport by overloading (see below) cannot be stated from these experiments.

Self-blockade of phenol red transport. At high phenol red concentrations in the medium, the transport of dye is blocked at step 2, with the consequence that the dye accumulates in the cell, with no detectable concentration in the lumen. (See first three columns of table 1.) (This situation is similar to that existing at high potassium, low calcium concentrations.) When the concentrations of phenol red and potassium are increased simultaneously, an additive effect is observed, in that cessation of excretion into the lumen and accumulation in the cell occur at lower concentrations of phenol red than are otherwise required to block tubular transport.

When the concentrations of phenol red and calcium are increased simultaneously, increasing concentrations of calcium release the self-blockade associated with high concentrations of phenol red.

TABLE 1

Effect of varying potassium concentration on accumulation of phenol red in cell

PHENOL RED CONCENTRATION IN MEDIUM	PHENOL RED CONCENTRATION IN MATCHING FILTER							
	2.5 mM/L K		10 mM/L K		20 mM/L K		30 mM/L K	
	Lumen	Cell	Lumen	Cell	Lumen	Cell	Lumen	Cell
mg %	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*
2	7.5	0	7.5	0	2.5	0	5	1.8
10	5	0	0	5	1.2	10	1.2	3.8
20	10	0	0	7.5	0.5	10	0.9	7.5
40	0	3.8	0	7.5	0.5	10	0.5	10
60	0	3.8	0	7.5	0.5	10	0.5	10

Effect of acetate. It is known that acetate increases the transport of PAH in rabbit kidney slices (Cross and Taggart, '50) and Tm_{PAH} in man (Baldwin and McLean, '52); and that the blockade in the tubular transport of this substance, which is encountered in the dog at high plasma concentrations, is reversed by acetate (Schachter and Freinkel, '51). Assuming similarity in transport mechanism, it might be anticipated that acetate would reverse the self-blockade in the transport of phenol red in the flounder tubule. However, acetate at concentrations of 5, 10 and 20 mM/L had no appreciable effect in this direction.

Action of the carbonic anhydrase inhibitor, Diamox (6063),[3] *on the reaction of the urine.* When the bicarbonate concentration of the medium is reduced from 20 to 0.625 mM/L, the apparent pH of the tubular urine decreases, until the dye has a color corresponding to about pH 6.6 — i.e., the urine becomes relatively acid in the presence of a low external bicarbonate concentration, despite the fact that the reaction of the medium itself remains alkaline.

---

[3] Supplied by the American Cyanamid Company, Stamford, Connecticut.

No precise method is available to determine the reaction of the urine except between the extremes of pH 6.6, where the dye has an intense yellow color, and pH 8.2 where it is intensely red. However, we have established two arbitrary, intermediate points of color change which we have designated as orange (ca. pH 6.6) and pink (pH 7.2) (fig. 1).

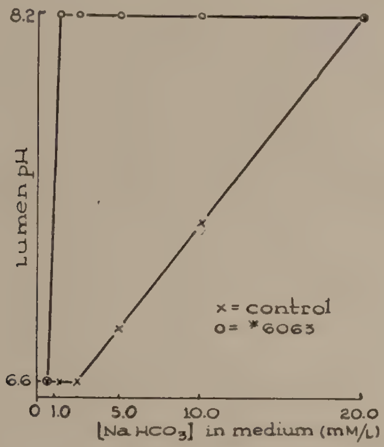


Fig. 1 pH of tubular urine at varying media concentrations of NaHCO$_3$ with (o) and without (x) no. 6063 in the media.

The carbonic anhydrase inhibitor, Diamox (2-Acetylamino-1, 3, 4 thiadiazole-5-sulfonamide) (Miller, Dessert and Roblin, '50) has been used by Berliner, Kennedy and Orloff ('51) to block acidification of the urine in mammals. This compound presumably works by inhibiting carbonic anhydrase in the renal tubules.

The effect of Diamox (1 mg %) on the flounder tubule was examined by observing the effects of decreasing bicarbonate concentration in the medium on the apparent pH of the urine (fig. 1). (It can be repeated that the medium was always alkaline, even at the lowest bicarbonate concentration.) In the presence of Diamox the tubular urine remained distinctly alkaline at an extreme bicarbonate concentration of 0.625 mM/L, where normally the urine would have a pH of 6.6. If we conceive that the progressive acidification of the urine, which occurs as the bicarbonate concentration of the medium is reduced, involves a carbonic anhydrase-sensitive, hydrogen-ion exchange mechanism, then the action of Diamox in preventing the acidification of the urine at low concentrations of bicarbonate can be referred to inhibition of this mechanism, as in the mammals.

If calcium is omitted from the medium, addition of Diamox causes the tubules to respond as though it were in a high potassium medium, i.e., dye accumulates in the cells, and the latter acquire an intense red color. This observation implies either that carbonic anhydrase is necessary for step 2, namely, the deposition of dye in the tubular urine, or an increase in alkalinity of the cells, as postulated by Berliner, Kenney and Orloff ('51), promotes absorption of the dye and/or intracellular accumulation.

DISCUSSION

The blockade of tubular excretion of phenol red which is associated with high concentrations of the dye, with high potassium concentrations, or with low calcium concentrations, is not to be confused with the self-depression of clearance which is associated with the elevation of the plasma concentration of all solutes excreted by the renal tubules: this latter phenomenon issues simply from the quantitative relations between load and Tm, and does not necessarily involve accumulation of solute (PAH, diodrast, phenol red) in the cell. On the other hand, the blockade in tubular transport described in the foregoing experiments represents cessation of tubular excretion into the lumen, under which conditions Tm must be depressed to or towards zero, as actually has been reported in the papers cited above.

Our present experiments show that, in this blockade, the three factors enumerated — high potassium and low calcium concentrations, and also high concentrations of dye — have an additive action, implying a common locus of action. This additive action is clearly evident in any one preparation (tables 1 and 2), but not reproducible with respect to critical concentrations of dye and potassium, in different preparations.

TABLE 2

Effect of varying calcium concentration on accumulation of phenol red in cell

PHENOL RED CONCENTRATION IN MEDIUM	PHENOL RED CONCENTRATION IN MATCHING FILTER					
	0.4 mM/L Ca		0.8 mM/L Ca		1.5 mM/L Ca	
	Lumen	Cell	Lumen	Cell	Lumen	Cell
mg %	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*
10	1.25	1.25	5	0	10	0
20	2.5	2.5	5	0	10	0
40	2.5	2.5	1.25	0	10	0
60	1.25	1.25	1.25	1.25	2.5	0
120	1.25	2.5	...	...	0	2.5

SUMMARY

The transport of phenol red in the isolated renal tubule of the flounder kidney has been examined (by visual observation) in media containing different concentrations of potassium, calcium and phenol red, and under the influence of Diamox (6063).

Previous observations have been confirmed that the dye is transported without cellular accumulation when the tubules are immersed in media balanced in the respect to calcium and potassium, but the dye accumulates in the tubule cells in media containing either high potassium or low calcium concentrations.

It is further shown that in balanced media, but at high phenol red concentrations, the dye accumulates in the cells, with marked reduction or complete arrest of excretion into the tubule lumen (self-blockade) : i.e. the last step in tubular transport seems to be blocked by overload. The effects of high potassium and low calcium concentrations, and of high phenol red concentrations, are additive.

The carbonic anhydrase inhibitor, Diamox (6063), enhances phenol red accumulation in the tubule cells, and, as judged by color, may increase the alkalinity of the cells. Diamox also prevents the acidification of the tubular urine which is characteristic of normal tubules in the presence of low concentrations of bicarbonate, implying that to some extent this acidification involves carbonic anhydrase, as in the mammals.

LITERATURE CITED

BALDWIN, D. S., AND P. G. MCLEAN 1952 Effect of acetate on Tm_{PAH} in man. J. Appl. Physiol., *4:* 797–799.

BARCLAY, J. A., W. T. COOKE AND G. DEMURALT 1949 Effect of increasing plasma levels on tubular excretion of diodone. J. Physiol., *108:* 8P.

BERLINER, R. W., T. J. KENNEDY, JR., AND J. ORLOFF 1951 Relationship between acidification of urine and potassium metabolism. Am. J. Med., *11:* 274–282.

CROSS, R. J., AND J. V. TAGGART 1950 Renal tubular transport: Accumulation of p-aminohippurate by rabbit kidney slices. Am. J. Physiol., *161:* 181–190.

EGGLETON, M. G., AND Y. A. HABIB 1950 Excretion of para-aminohippurate by the kidney of the cat. J. Physiol., *110:* 458–467.

ENGSTRÖM, A., AND B. JOSEPHSON 1953 Historadiographic demonstration of diodrast in the rabbit kidney. Am. J. Physiol., *174:* 61–64.

FORSTER, R. P. 1948 Use of kidney slices and isolated renal tubules for direct study of cellular transport kinetics. Science, *108:* 65–67.

FORSTER, R., AND J. TAGGART 1950 Use of isolated renal tubules for the examination of metabolic processes associated with active cellular transport. J. Cell. and Comp. Physiol., *36:* 251–270.

JOSEPHSON, B. 1949 The mechanism of renal tubular excretion. Scand. J. Clin. Lab. Invest., *1:* 344.

——— 1950 Studies on renal tubular excretion in the rabbit. Abstracts of XVIII International Physiol. Congress: 281.

JOSEPHSON, B., AND J. KALLAS 1953 The iodine concentrations in rabbit kidneys after diodrast injection. Am. J. Physiol., *174:* 65–71.

MILLER, W. H., A. M. DESSERT AND R. O. ROBLIN, JR. 1950 Heterocyclic sulfonamides as carbonic anhydrase inhibitors. J. Am. Chem. Soc., *72:* 4893–4896.

PUCK, T. T., K. WASSERMAN AND A. P. FISHMAN 1952 Some effects of inorganic ions on the active transport of phenol red by isolated kidney tubules of the flounder. J. Cell. and Comp. Physiol., *40:* 73–88.

SCHACHTER, D., AND N. FREINKEL 1951 Self-depression of Tm_{PAH} in the dog at high plasma PAH levels and its reversibility by acetate. Am. J. Physiol., *167:* 531–538

STUDIES ON ARTHROPOD CUTICLE

X. THE ASYMMETRICAL PENETRATION OF WATER [1, 2]

A. GLENN RICHARDS, MARION B. CLAUSEN
AND MYRTLE N. SMITH
*Department of Entomology and Economic Zoology, University
of Minnesota, St. Paul*

EIGHT FIGURES

An insect cuticle is a heterogeneous, secreted, non-cellular membrane, the outer sublayer of which (epicuticle) differs in physical and chemical properties from the inner (endocuticle). If an isolated sheet of insect cuticle is used to separate fluid water from dry air, the rate of penetration of water is said to be several to many times as rapid if the inner surface of the cuticle faces air than if the outer surface faces the air. The phenomenon is termed ''asymmetrical penetration,'' and the ratio of the rates in the two directions is the ''asymmetry value.'' The asymmetrical penetration rates recorded for water by several workers (Hurst, '41, '48; Beament, '45) in water : cuticle : air systems is assumed to be due to the asymmetry of the system, not to the visible asymmetry of the cuticle (see Symposium, 1948). If this assumption is correct, the phenomenon should not be encountered in a water : cuticle :

---

[1] Paper No. 2900, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul 1.

[2] The work described in the present paper was done under terms of a contract between the Office of the Surgeon General, U. S. Army and the University of Minnesota.

Our thanks are due to Dr. O. H. Schmitt of the Biophysics Division for reading this manuscript, to Dr. M. Cavert of the Physiology Department of the University of Minnesota Medical School for instruction in the technique of D_2O measurement, and to Mr. J. M. Saari for the mass spectrometer determinations under the supervision of Dr. A. O. Nier of the Physics Department.

water system. This is here shown to be correct by means of tests employing heavy water as a tracer. The tests were also performed to determine whether the high asymmetry values of Hurst or the low ones of Beament were repeatable. And, more importantly, the tests were performed to see if consideration of the rates of water movement under different experimental conditions would aid in selection or elimination among the various mechanisms postulated by Hurst, Beament, Hartley, Barkas and Klinkenberg (Symposium, 1948).

METHODS

Cuticles were isolated from last instar larvae of *Sarcophaga bullata* which had been reared on a diet of ground beef at 25°C. The larvae for these tests were reared specially in glass jars under conditions where it seems impossible for them to have become scratched during growth. Larvae were found to give similar results throughout 3–6 days of age.

Isolated membranes were prepared by splitting last instar larvae along the middorsal line, eviscerating, then soaking in distilled water at 5°C., usually overnight, and gently swabbing the inner surface of the cuticle while immersed in distilled water. The swabbing was done with a small sable brush on the surface of clean wax. The cuticles were passed through several changes of distilled water, with repeated swabbing, until the inner surface appeared clean under a dissecting microscope. Tests showed that it was immaterial whether the cuticles remained in distilled water in the refrigerator for one hour or one or two days. It was also found to be immaterial whether the cleaning in distilled water was at 5°C. or at room temperature. For lipid extraction, cleaned cuticles were passed through acetone to chloroform, refluxed for two hours, and returned to water through acetone; they were not allowed to dry.

For the evaporation tests with H_2O and D_2O, membrane holders similar to those used by Beament ('45) were employed. We found that more constant and repeatable results were obtained with cells, including membrane holders, made entirely of glass and sealed with a beeswax-resin mixture. Sealing with the beeswax-resin has the additional advantage of permitting the mounting of wet membranes in a water-saturated atmosphere as well as the mounting of dry membranes. Successful inversion of individual membranes is facilitated by sealing the cuticles between two perforated glass plates; one can then reverse the whole sandwich without dismounting the cuticle. These cells had evaporating areas of 14.2 mm.^{2}

For the test periods, the evaporating cells were placed in desiccators over P_2O_5 or $CaCl_2$ (0% R.H.) or over saturated solutions of $MgCl_2$ (33% R.H.), K_2CO_3 (43% R.H.), CrO_3 (44% R.H.), NaBr (58% R.H.), NaCl (75% R.H.) or KBr (81% R.H.). These solutions were stirred periodically. In the first experiments (figs. 1–2 and table 2) no provision was made for stirring air within the vessels; in later experiments circulation was induced by unilateral heating which gave a 2°C. differential between the two sides of the 8-inch vessel (figs. 3–5). More consistent results are obtained over a period of days if changes in hydrostatic pressure are avoided; tests showed that capillary openings from the cells did not permit significant loss if the diameter of the capillary was less than 0.1 mm.

A minor number of cuticles showed wide and erratic fluctuation from day to day. Such data were discarded. We retained as satisfactorily consistent all membranes the data from which did not vary from day to day (4-day period) by more than 2 × when the epicuticle faced air and which did not increase in rate when the endocuticle faced air.

Evaporation of water from intact larvae (transpiration) was measured with KCN-killed larvae since Wigglesworth ('45) has shown that rates are similar from dead insects and living ones with occluded spiracles. Abrasion was performed by dragging intact larvae across 2/0 emery cloth under pressure of an overlying finger.

D_2O penetration rates in water : cuticle : water systems were made by clamping cuticles between lucite half-cells which had a volume of 1.4 ml. on each side and an exposed membrane area of 19.6 mm.[2] A glass bead which could roll but not hit the membrane was built into each half-cell. Movement of the beads during slow shaking kept the solutions stirred. One per cent excess D_2O by volume was used on one side of the cell (99.8% pure, from Stuart Oxygen Co.). Tests were run for 72 hours at 20°C. The concentration gradient did not change significantly during the test (2–6% difference at end).

Even though the half-cells were filled, emptied, rinsed, and refilled without removing the membrane, it was still found necessary to make three measurements on each membrane in order to eliminate seals or membranes which had become defective. These measurements were an initial one with the D_2O moving in one direction, a second measurement with the D_2O moving in the opposite direction through the same membrane, and a third measurement which was a repeat on the first. D_2O was measured in samples from each side of the cell; recovery was 96–100%. The hydrogen equilibration method was used with determination of the HD/H_2 ratio by a mass spectrometer (Kirshenbaum, '51, p. 205–233).

RESULTS

Penetration in water:cuticle:water systems

The evaporation tests reported in the next section show that the penetration values for D_2O are of the same magnitude as the values for H_2O. Therefore heavy water is a satisfactory tracer for cuticle permeability work. In a set of 6 membranes tested after standardizing the technique, 4 gave the same value within experimental error on repetition of the first test. Values for these are given in table 1. The average for the two directions was identical! Therefore there is no asymmetry in penetration when liquid water is present on both sides of the cuticle. Accordingly there cannot be molecular valves of a gate-valve type in the cuticle.

The 4 membranes listed in table 1 show a 3–4 × individual variation. This is to be expected in view of the higher individual variations for penetration of electrolytes through fly cuticles reported by Ricks and Hoskins ('48) and by Richards and Fan ('49). It may be noted in passing that the absolute rate is very much less (about 2000 × less) than the average rates cited for passage of water through plasma membranes (Davson and Danielli, '43), and of the same magnitude as the value for penetration of KCl through the cuticle of another fly, *Phormia regina* (Richards and Fan, '49).

Penetration in water:cuticle:air systems

When the epicuticle side is against air, similar values are obtained irrespective of whether the membrane was mounted wet or dry. The two dotted curves of figure 1 represent extreme averages of single runs. The average of all 33 cuticles tested in this series lies midway between these two curves. Values were similar for the penetration of H_2O and D_2O. Values for water loss through isolated cuticles in this orientation were closely similar to the average transpiration rates of 44 doubly ligated larvae in dry air; therefore tests with these isolated membranes would appear to be quite valid. Incidentally, these values are similar to the transpiration values reported by Wiggleworth ('45) for larvae of another fly, *Calliphora erythrocephala.*

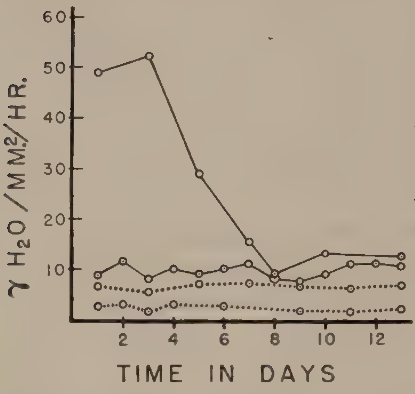
TABLE 1

Permeability values for heavy water in water: cuticle: water system.

Values expressed as μMoles/mm²/hr./mole difference in concentration

DIRECTION	MEMBRANE NO. 1	MEMBRANE NO. 2	MEMBRANE NO. 3	MEMBRANE NO. 4	AVERAGE
Epicuticle to endocuticle	0.0139	0.0540	0.0197	0.0185	0.0265
Endocuticle to epicuticle	0.0212	0.0540	0.0139	0.0167	0.0265

When the cuticle is mounted with the endocuticle side against air, the penetration rate of water is consistently higher. If the cuticles were air-dried during mounting, average asymmetry values of 2 × were obtained for H_2O and 3 × for D_2O (lower solid lines of figs. 1–2). It is concluded that an average asymmetry value of 2–3 × is the true difference in this species when the membranes have been thoroughly air-dried during mounting. If, however, the cuticles were mounted in a humid atmosphere and accordingly not allowed to lose their moisture prior to being placed in the desiccators, variable results were obtained. Some individual curves and the average of curves for D_2O penetration tests were nearly flat and not significantly different from values given by air-dried cuticles (top solid line of fig. 2). In more than half of the cases summarized in table 2, however, undried cuticles gave initially high values which fell with time almost but not quite to the level given by air-dried cuticles (top solid line of fig. 1). With more experience and care initially high asymmetry values were nearly always obtained (figs. 4–5). We conclude that the asymmetry value for this species when the



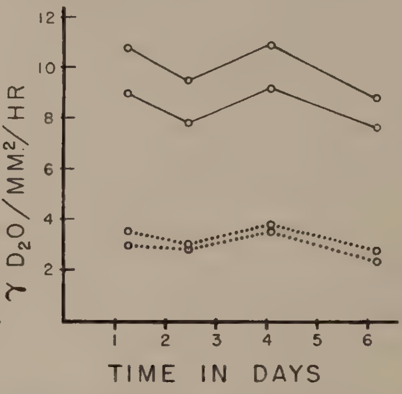


Fig. 1 Rate of evaporation of water through isolated cuticles of *Sarcophaga bullata* in a water: cuticle: air system at 20°C. The air was dry and was not stirred. Solid lines, endocuticle facing air; dotted lines, epicuticle facing air. Points on the top curve represent averages from 11 cuticles, on the second curve averages from 34 cuticles, while the bottom two curves represent extremes of series totaling 51 cuticles. See text.

Fig. 2 Same but for heavy water. Each of the 4 curves is based on averages from 5–6 cuticles. See text.

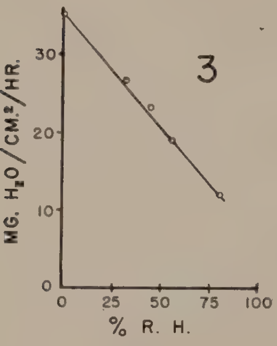
membranes are mounted wet ranges from 5 × to 25 × (averaging about 10 ×) at the start decreasing within a week to 3–5 ×. In a few exceptional individual cases initial asymmetry values of 30 × and even 40 × were obtained.

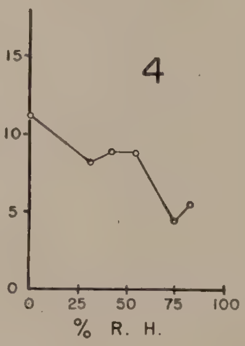
Individual asymmetry values are not available for the cuticles air-dried before mounting since different ones were used to measure the rate in the two directions. Average values for these can be seen in figures 1 and 2. In later experiments with wet-mounted cuticles, individual ones were routinely reversed (figs. 4–5). Individual variation in actual penetration rate within a test lot is usually over 10 × when the epicuticle faces air, and for the most exceptionally high and low values encountered reaches an extreme of about 50 ×. Similar individual variation in rate of salt penetration has already been reported (Ricks and Hoskins, '48; Richards and Fan, '49). In contrast, individual variation in actual rate on the first day for wet-mounted cuticles with the endocuticle facing air was always less than 10 ×, usually 4–6 ×. Low individual asymmetry values of between 2 × and 10 × were always associated with relatively high penetration rates for that membrane when the epicuticle was against air. Conversely, high individual asymmetry values of 20 × or more were associated with a lower than average penetration rate when the epicuticle was against air. However, a low rate when the epicuticle faces air does not necessarily mean that the cuticle is going to give a high asymmetry value.

Our asymmetry values from air-dried membranes are in good agreement with the values reported by Beament (Symposium, 1948) whose cuticles were always air-dried during mounting. Our asymmetry values for membranes mounted while wet were initially considerably higher than the values given by Beament for air-dried membranes but still considerably lower than the asymmetry value of 100 × reported by Hurst for cuticles mounted while wet. Actually, Hurst ('48) reported these very high asymmetry values only for measurements made within 20 minutes after mounting the membranes; his asymmetry values at the end of an hour are comparable to asymmetry values obtained at the end of 24 hours in the present experiments. However, the absolute values in terms of mg/cm²/hr. reported by Hurst for cuticles from larvae of *Calliphora* were several times higher than the highest values we obtained with cuticles of *Sarcophaga.*

Penetration as a function of humidity

If the asymmetry phenomenon has a simple physical explanation one might expect a linear relationship between evaporation rate and relative humidity of the gas phase. Accordingly, measurements were made at 6 humidities. No difficulty was encountered in obtaining an approximately straight line relationship between evaporation from a free water surface and humidity (fig. 3). The fact that this line extrapolates to zero somewhat beyond 100% R.H. is to be expected since the tests had to be run in closed vessels in





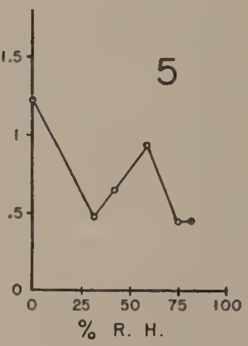


Fig. 3 Rate of evaporation of water from a free water surface of same area as aperture in membrane holders plotted as a function of relative humidity. Experiment run at 26°C. with thermal stirring of air. Control for figures 4–5.

Fig. 4 Rate of evaporation of water through isolated wet-mounted cuticles of *Sarcophaga bullata* with the endocuticle facing thermally stirred air of the indicated relative humidities at 26°C. Each point represents the average of determinations after one day on 10–15 membranes except the values at 75% and 81% R.H. where only 2–6 membranes remained consistent.

Fig. 5 Rate of evaporation through the same cuticles as used for the preceding but with the epicuticle facing thermally stirred air. These determinations were made immediately prior to those plotted in figure 4.

a dry laboratory, and the vessels had to be opened at each weighing. The periodic mixing with laboratory air (about 25% R.H.) requires re-equilibration in the gas phase; this would depress slightly the left end of the curve while raising slightly the right end.

Figures 4 and 5 are not straight lines, and in percentage values do not agree with percentage decreases in the control (fig. 3). Obviously the state of the cuticle depends on the humidity of the gas phase and deviates from the control most in the range of 40–60% R.H. Correlated with the fact that circulation of air was induced in these tests (also temperature slightly higher), the numerical values at 0% R.H. are somewhat more than twice those given for wet-mounted cuticles in the preceding section (fig. 1). The asymmetry ratios, however, remain about the same, at various R.H. values ranging from averages of 8 × to 16 ×. The range of asymmetry values of individual membranes at the various R.H. values appears to be similar (low, intermediate and high values occur in the sets at each humidity).

It is not known whether a single cuticle tested at each humidity successively would give these same curves. The action of contaminating bacteria leads to alteration of rate after about a week at 25°C. Accordingly, different sets of cuticles were used at each humidity in our tests so that we could check over a period of days in order to be able to eliminate cuticles that did not give consistent results and yet not encounter serious bacterial trouble. Since the individual cuticles were all reversed, values at any particular R.H. in figure 4 are from the same membranes as values at that R.H. in figure 5.

Penetration through treated cuticles

For analysis of the role of subdivisions of the cuticle it is possible to remove or alter certain portions without demonstrably changing others (Wigglesworth, '45; Richards, '51). Isolated cuticles were exhaustively extracted with boiling chloroform, and then 8 measured at each of 5 humidities.

The data did not differ significantly either in actual rates or asymmetry values from those given in the preceding section. No essential part of the responsible structure is readily removed from the cuticles of fly larvae by extraction with chloroform.

Isolated cuticles were extracted for 20 minutes in distilled water at 68° and 82°C. These treatments should remove most of the soluble protein arthropodin from the endocuticle and induce reorientation in at least the chitin fraction of the endocuticle (Richards, '51). In the few membranes tested results were variable and will not be given in detail. However, clearly the asymmetry value of approximately 10 × for wet-mounted membranes was not noticeably changed. Actual penetration rates after treatment at 68°C. (4 cuticles) seemed about normal; others after treatment at 82°C. (4 cuticles) were approximately twice as high.

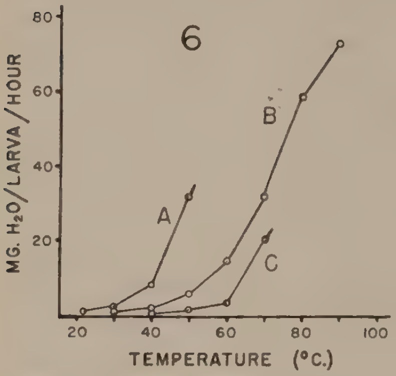


Fig. 6 Curve *B* shows the average transpiration rate from 20 KCN-killed *Sarcophaga* larvae in a $CaCl_2$ desiccator at various temperatures. Each larva had a surface area of approximately 2 cm^2. For comparison, curves *A* and *C* are replotted from Wigglesworth's data for larvae and puparia, respectively, of *Calliphora erythrocephala.*

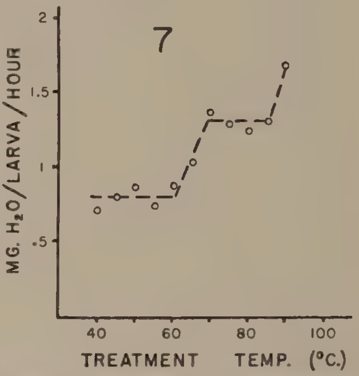


Fig. 7 The average transpiration rate over $CaCl_2$ of KCN-killed *Sarcophaga* larvae at 25°C. after being heated in air at the indicated temperatures for 20 minutes. Each point represents an average from 8 larvae. Each larva had a surface area of approximately 2 cm^2. Deviations of points from the line drawn are within experimental error.

Transpiration tests where water loss was measured as a function of temperature gave results similar to those already reported by Wigglesworth ('45) for the fly *Calliphora* (fig 6). The "breaking point" in the curve for *Sarcophaga* is less sharp and lies between that for the *Calliphora* larva and puparium. Apparently one has to go fairly high up on this curve before irreversibly altering these fly cuticles. Treatments at temperatures up to something over 60°C. do not result in increased rates when transpiration is subsequently measured at 25°C. (fig. 7). Treatments above 70°C. approximately double the rate. Some may prefer to draw a sigmoid curve through the points in figure 7; it does not matter for the present discussion. Drawing a sloping straight line through the points would increase the scatter to more than twice the ascribable experimental error. These data are in general agreement with those from heat-treated isolated cuticles given in the preceding paragraph. This is indeed a small change in comparison to the change in cuticles sufficiently abraded to have lost their asymmetry.

Attempts to abrade the outer surface of the cuticle of *Sarcophaga* larvae by rubbing with 120 mesh emery powder gave negative results both in terms of transpiration measurements and microscopic examination. Apparently the cuticle of fly larvae is much more difficult to abrade than that of most insects (Wigglesworth, '45). Lightly rubbing across emery cloth likewise gave negative results. Rubbing across emery cloth with either moderate or heavy pressure did result in great increases in transpiration rates (10–20 × or more). Such larvae immersed in solutions of acid fuchsin, black Sudan B or ammoniacal silver nitrate show stained microtrichia and stained scratches. A photograph of an abraded cuticle is shown in figure 8. Cuticles showing only the fine scratches have the transpiration rate raised only a few times and still show asymmetry; cuticles showing the broad scratches have the transpiration rate raised over 10 × and have lost their property of asymmetry. Sections prepared from such cuticles show the tips of microtrichia absent and stained. They also show stained scratches of various depths; the fine scratches appear to be in the epicuticle but the broad bands involve deep cuts which penetrate all the way through the epicuticle for at least part of their course.

After determining individual transpiration rates, then scratching and re-determining the transpiration rate, cuticles were isolated and mounted, taking care that the area over the perforation in the holder was an area that had been abraded. A total of 20 cuticles of known transpiration change were measured for asymmetry. Those in which the transpiration rate had increased 10 × or more showed correspondingly high penetration rates and no asymmetry. Those in which the transpiration rate had increased only 4–8 × still showed asymmetry but at a lower ratio. It seems, then, that to eliminate asymmetry deep cuts through the epicuticle are necessary.

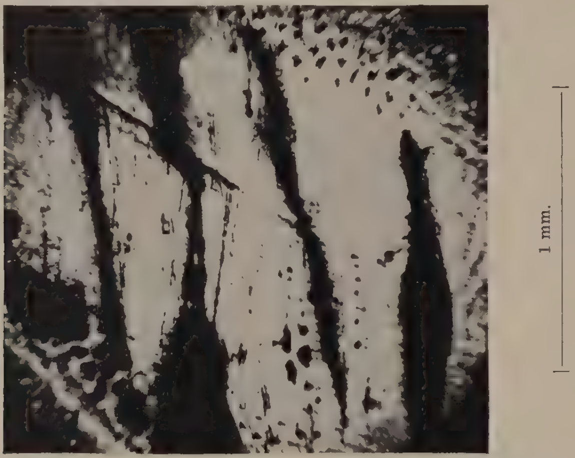


Fig. 8 Photograph of a portion of the abraded cuticle isolated from a larva of *Sarcophaga bullata* after soaking overnight in ammoniacal silver nitrate. Shows fine scratches, heavy deep scratches which appear as broad bands, and spots which are abraded tips of microtrichia.

Since abrasion with emery cloth not only makes visible scratches but presumably abrades most of the surface lightly, a further experiment was performed. Seven isolated cuticles were scored each with 4 deep scratches with the broken tip of a glass needle. These scratches cut through the epicuticle of some small percentage of the exposed area (certainly less than 10%). The rate of evaporation through these cuticles was increased as much as by heavy abrasion with emery cloth, and the asymmetry was lost.

From these tests it is concluded that the epicuticle is essential to the asymmetry phenomenon in this species, that the essential constituents are not significantly altered by hot chloroform or hot water, and that the inner portion of the epicuticle (cuticulin layer or inner epicuticle, Dennell and Malek, '53) is essential and may even be the controlling locus in fly larvae.

Desiccation of one species of insect by another species

Hurst ('41) also made the curious report that whereas larvae of the fly *Calliphora* and the beetle *Tenebrio* both withstand desiccation in a dry atmosphere for a long time, the *Tenebrio* larva dries rapidly if confined in contact with a *Calliphora* larva. He postulated that this is due to the lipid of *Calliphora* being more hydrophilic than that of *Tenebrio.*

We tested this using various sizes of larvae of *Tenebrio molitor* confined in contact with larvae of the flies *Calliphora erythrocephala, Sarcophaga bullata* and *Phormia regina.* A total of 71 pairs plus controls were confined in contact in a desiccator by wrapping in clean silk bolting cloth. They were checked after 24 and 48 hours.

Highly erratic results were obtained in the first sets of tests which were deliberately somewhat diversified. The controls of the initial series showed that fly larvae withstood relatively rough manipulation but were more sensitive to high temperature; *Tenebrio* larvae were easily killed if mishandled. One can repeat Hurst's results if the larvae are consistently treated roughly, but careful handling is followed by both larvae remaining alive and apparently quite normal. We conclude that there is not true desiccation of one species by the other.

DISCUSSION

Values from various tests are listed in table 2. Comparison of the values in this table show that the H_2O and D_2O loss rates to dry air do not agree quantitatively with D_2O penetration rates when liquid water is present on both sides of the cuticle. Line 3 is only 7.5 × line 1, yet the gradient of liquid water to dry air is equal to a 55 mole concentration difference. Later experiments where the air was stirred (figs. 4–5) could raise this by no more than a factor of two. Therefore the cuticle cannot be considered the same for penetration values in water : cuticle : water and water : cuticle : air systems. In one case, viz., the initial values for wet-mounted membranes (line 6a) corrected for concentration gradient, a seeming approximation to values for cuticles with liquid water on both sides can be obtained. But in later experiments where the air was stirred, values approximately 2 × these were obtained.

Suggestions that have been or might be advanced to account for the phenomenon of asymmetrical penetration through insect cuticles are: (1) That there is some form of molecular gate-type valve in the cuticular pores which opens when water passes in one direction but partially closes when water goes in the other direction. (2) That water molecules partially clog pores of fixed dimension so that the cuticle is less permeable when the endocuticle is wet than when it is dry (Barkas, in Symposium, p. 223). (3) That due to the relatively hydrophobic nature of the surface of the epicuticle, the actual surface area for evaporation is different by the asymmetry value (Beament, in Symposium, p. 221). Or, in more detail, when the epicuticle side of the cuticle is against air evaporation is limited by the area of pores through this layer, but that when the endocuticle is against air fluid water is drawn through these pores and evaporates from the entire surface of the cuticle. (4) That there are molecular or at least micro valves in the epicuticle of a type that swell open by imbibition when

TABLE 2

Penetration rates for larval cuticles of Sarcophaga bullata at 20°C. Includes data only from experiments where circulation of air was not induced within the containers

SUBSTANCE	SPECIFICATIONS	RANGE	AVERAGE	UNITS
1. D_2O penetration	both sides wet	0.014–0.054	0.0265	μM/mm^2/hr./mole conc. difference
2. D_2O evaporation	epicuticle against air**	0.12 –0.23	0.16	μM/mm^2/hr./water vs. dry air
3. H_2O evaporation	epicuticle against air**	0.05 –0.35	0.20	μM/mm^2/hr./water vs. dry air
4. D_2O evaporation	endocuticle against air**	0.33 –0.73	0.49	μM/mm^2/hr./water vs. dry air
5. H_2O evaporation	endocuticle against air, dried	0.13 –1.0	0.40	μM/mm^2/hr./water vs. dry air
6. H_2O evaporation	endocuticle agains air, not dried			
(a)	initial	0.2 –4.5	1.0–2.7*	μM/mm^2/hr./water vs. dry air
(b)	falling with time to	0.2 –1.6	0.7	μM/mm^2/hr./water vs. dry air
7. H_2O evaporation	transpiration of intact insect	0.06 –0.31	0.17	μM/mm^2/hr./body fluid vs. dry air

* Averages from two sets of five membranes from different lots of larvae tested at different times. These could be averaged to give a value of 1.8.

** Dry-mounted and wet-mounted membranes combined in these lines.

the epicuticle is against water and shrink when the epicuticle is partially dried at an air interface (Hurst, '48). (5) That a complicated mathematical function of dry composition and swelling will result in asymmetry in a system of superimposed layers of different composition (Hartley, in Symposium, p. 223).

The first of the above suggestions is entirely discredited by the finding that there is no asymmetry in a water: cuticle: water system (table 1). The second is discredited by finding that maximal penetration rates are given by wet cuticles and minimal ones by dry cuticles (table 2). The third is discredited both by the non-linear relation between evaporation rates and relative humidity (figs. 4–5) and by the finding that deep scratches which destroy less than 10% of the epicuticular surface abolish the asymmetry. The 4th is difficult to reconcile with the decreasing penetration rates of wet-mounted cuticles (top curve, fig. 1); this would necessitate the postulated valves shrinking rapidly when the epicuticle faces air and yet slowly when the epicuticle is in contact with fluid water. The 4th suggestion is discredited by the same points which discredit the third suggestion. It cannot be made to explain the hump in figures 4 and 5 except by modification which makes it in effect a special type of mechanism for the 5th suggestion. The 5th suggestion is the only one which can be made to fit all the data in the present paper.

As a modification of the usual permeability equation, Hartley says, "... we can easily show that if x is distance through a membrane and c is concentration, having uptake and exit values at O and X of c_2 and c_1, the rate of transfer per unit cross section G is given by

$$G = -D\frac{dc}{dx} = -f\,(x, c)\,\frac{dc}{dx}$$

in the general case." If the complicated function $f\ (x, c)$ is separable as $f(x) \times f(c)$ the membrane will behave symmetrically, but when, as usual, the complicated function is not separable the membrane will behave asymmetrically. Further elaboration of this scheme could conceivably explain all of the data. However, although a complicated function seems to be required, we have no assurance that x and c really have their stated meanings. And there seems little likelihood of making the determinations necessary to identify x and c and to elaborate Hartley's general equation. Accordingly, the equation is a statement of necessary and sufficient conditions in general terms rather than a specific explanation of the particular case in insect cuticles.

Attempts to localize the important components and subdivisions were only partially successful. No essential part is removed by either hot water or hot chloroform, but neither of these treatments seem to alter the highly polymerized lipids and lipoproteins of the epicuticle (Dennell and Malek, '53). Penetration rates may be permanently raised some 2–3 × by heat treatment without altering the asymmetry value noticeably. Abrasion which raises the transpiration rate only a few times may lower but does not abolish the asymmetry; abrasion which raises the transpiration rate 10–20 × or more does abolish asymmetry. Histological examination suggests that when the scratches cut completely through the epicuticle asymmetry is lost. Perhaps this means that the cuticulin layer in combination with an endocuticular substratum is responsible for the asymmetry in cuticles of fly larvae but proof is lacking. One reservation should be kept in mind in connection with the abrasion data presented herein. Namely, the epicuticular surface of these larvae is not strongly hydrophobic; a few scratches affecting only a small percentage of the surface area might not abolish asymmetry in species with pronouncedly hydrophobic epicuticular surfaces.

It seems doubtful that this phenomenon has any important significance in the life of an insect since these animals never turn their skins inside out. Clearly, the asymmetry phenomenon is not of a sort which can operate either to accumulate or conserve water. At most, those insects, such as fly larvae, which live normally either in liquid media or exposed to air would have a higher penetration rate for water leaving through the cuticle when they are in an aqueous medium. But at its highest the penetration rate through the cuticle is still very low, and the osmotic pressure gradients would usually be operating in the opposite direction.

SUMMARY

1. Asymmetrical penetration values of 2–3 × are given for water and heavy water by isolated larval cuticles of the fly *Sarcophaga bullata* in a water : cuticle : air system if the membranes are air-dried during mounting. Asymmetry values of 5–25 ×, averaging 10 ×, and falling with time to 3–5 ×, are given if the cuticles are mounted wet and measured over a period of a week.

2. No asymmetry is shown in penetration rates when liquid water is on both sides of the isolated cuticles.

3. Evaporation rates through isolated cuticles plotted as a function of relative humidity do not give a straight line. They show too high rates in the range of 40–60% R.H. The asymmetry values are independent of humidity.

4. Asymmetry in rates is abolished by deep abrasion of the epicuticle but not by extraction with hot water or hot chloroform or high heat treatments in air.

5. Some permanent elevation (2–3 ×) of transpiration rates can be induced by heat treatments but much greater changes (10–20 ×) are induced by abrasion which cuts through the epicuticle.

6. Integrity of the inner epicuticle (cuticulin layer) seems essential for the phenomenon of asymmetry in cuticles of these larvae.

7. Of the explanations suggested for the asyemmetry phenomenon, the data are consistent only with the hypothesis that the penetration rate is controlled by a complicated function of two or more variables.

8. The permeability value for heavy water when the larval cuticles of *S. bullata* are wet on both sides averages 0.0265 μM/mm^2/hour/mole concentration difference. This is *ca* 2000 × lower than erythrocyte values. Lower permeability values are obtained if either side of the cuticle is dry, and the aver-

age transpiration rate in dry air, corrected for concentration, is almost 10 × lower. In effect, these cuticles are membranes whose properties change with the environment.

9. The report that one of two insect species in bodily contact can dehydrate the other is discredited.

LITERATURE CITED

BEAMENT, J. W. L. 1945 The cuticular lipoids of insects. J. Exp. Biol., *21:* 115—131.

DAVSON, H., AND J. F. DANIELLI 1943 The Permeability of Natural Membranes. Cambridge Univ. Press.

DENNELL, R., AND S. R. A. MALEK 1953 Homology of the layers of the epicuticle of insects. Nature, *171:* 298.

HURST, H. 1941 Insect cuticle as an asymmetrical membrane. Nature, *147:* 388–389.

——— 1948 Asymmetrical behaviour of insect cuticle in relation to water permeability. Disc. Faraday Soc., no. 3, p. 193–210.

KIRSHENBAUM, I. 1951 Physical Properties and Analysis of Heavy Water. McGraw Hill.

RICHARDS, A. G. 1951 The Integument of Arthropods. Univ. Minnesota Press.

RICHARDS, A. G., AND H. Y. FAN 1949 Studies on arthropod cuticle. 5. The variation in permeability of larval cuticles of the blowfly, Phormia regina. J. Cell. and Comp. Physiol., *33:* 1–22.

RICKS, M., AND W. M. HOSKINS 1948 Toxicity and permeability. 2. The entrance of arsenic into larvae of the fleshfly, Sarcophaga securifera. Physiol. Zool., *21:* 258–272.

SYMPOSIUM 1948 Interaction of water and porous materials. Disc. Faraday Soc., no. 3, p. 169–239.

WIGGLESWORTH, V. B. 1945 Transpiration through the cuticle of insects. J. Exp. Biol., *21:* 97–114.

THE BIOSYNTHESIS OF STABLE COBALTO-PROTEINS BY PLANTS

II. INTERACTION OF IRON AND COBALT METABOLISM IN NEUROSPORA CRASSA [1]

ROBERT BALLENTINE
McCollum-Pratt Institute, The Johns Hopkins University, Baltimore

FOUR FIGURES

In a former publication (Ballentine and Stephens, '51) it was demonstrated that in plants and microorganisms, especially *Neurospora crassa,* the metabolism of cobalt involves its incorporation into stable protein complexes. By exchange and complexing reactions it was shown that these complexes were non-ionic in nature, and that they were formed only during the growth and metabolism of the organism. Their protein nature was construed from their precipitability by trichloroacetic acid and their failure to dialize. At that time the question was raised that, while the level of this metabolism is of the order of magnitude of vitamin B_{12} metabolism in other organisms *(Euglena gracilis),* there exists the possibility that the cobalt is being metabolically incorporated into the cytoplasm in place of iron by a metabolic mistake. The experiments reported in this paper were set up to test this point, and by means of covariance analysis of the data, such a metabolic mistake seems very improbable. It appears that the metabolism of cobalt pertains directly to this element and not to iron.

---

[1] This is contribution no. 47 from the McCollum-Pratt Institute. This work was supported in part by a contract, AT(30-1)-933, between the Atomic Energy Commission and the The Johns Hopkins University.

EXPERIMENTAL

The methods presented previously (Ballentine et al., '49) were those used in these experiments with the exception of the culture methods.

Culture methods. The pink conidial wild type strain 5297A of *Neurospora crassa* was replaced by the albino wild type strain 15300a. The mold was grown in 250 ml of medium in 1 L Erlenmeyer flasks at 30°C. The medium was the same as that used previously, except for the nitrogen source. The ammonium nitrate (12.5 mM N/L) was replaced by a nitrogen-equivalent amount of ammonium acetate (1.92 gm/L). This media shows a much smaller change in pH during growth, and the single nitrogen source permits the use of this data in other studies to be reported at a later time.

RESULTS AND DISCUSSION

In any experiment involving the complex phenomena of growth and the response to variables both correlated with and effective upon the rates of growth, a factorial design is essential to allow statistical analysis of the results. The experiments to be reported were designed and the analysis of variance performed with the very welcome advice of Dr. A. E. Brandt. The experimental parameters are (1) the length of growth, (2) the iron concentration and (3) the cobalt concentration.

The values selected are as follows:

(1) Time, days	(2) Iron, μM/L	(3) Cobalt, μM/L
4	0.355 (1/10 Fe)	7×10^{-3} (1 × Co)
7	3.55 (1 × Fe)	7×10^{-2} (10 × Co)
10 (11)	35.5 (10 × Fe)	7×10^{-1} (100 × Co)
14		

The experiments were run in all possible combinations of these parameters, giving 36 sets of data. Each set consisted of the following observations: (a) total weight of mold/L, (b) mg protein/100 mg dry mycelium, (c) μM total cobalt accumulated/100 mg dry mycelium and μM/L, and similarly for (d) stably-bound soluble cobalt, and (e) stably-bound particulate cobalt.

Figure 1 shows one set of data obtained with 10 × Co at the three iron concentrations. The values given are for the total

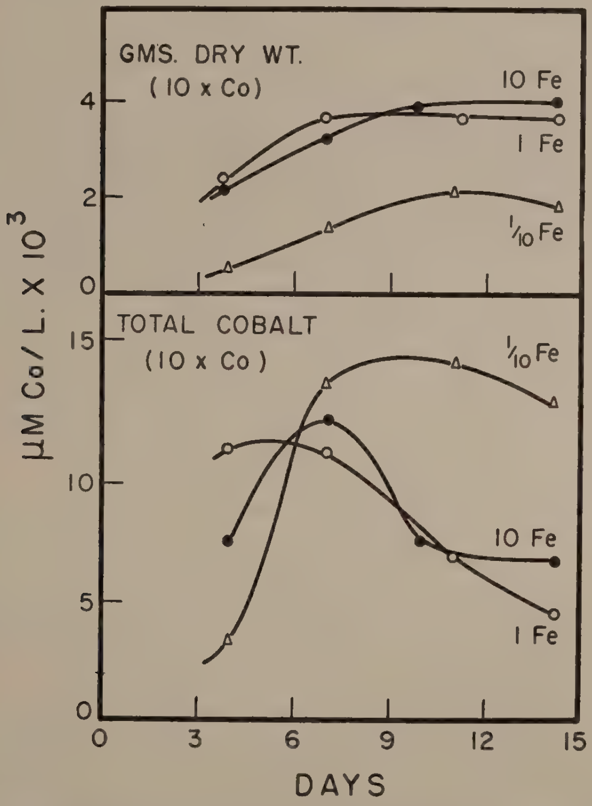


Fig. 1 *Neurospora* growth and total cobalt accumulation in media containing normal, 1/10th and 10 × normal iron concentration.

Ordinate: Upper curves, gm dry weight mycelium formed in 1 L of culture. Lower curves, $\mu M \times 10^3$ total cobalt accumulated by the mycelium in 1 L of modified Fries medium at a cobalt concentration of 7×10^{-2} μM/L.

Abscissa: Days of growth at 30°C. in standing cultures, 250 ml medium per liter flask.

cobalt accumulated/L of culture. Inspection of this figure shows a clear inverse effect of the iron concentration on cobalt accumulation. However, the dry weight curves, also shown in figure 1, show that this effect of iron is not a simple one on cobalt metabolism alone, but that other metabolic patterns are probably changed with the change in iron level.

TABLE 1

Analysis of variance

Entries in the table are for F, except in the case of *error* where the mean squares are given.

Symbols: D of F = degrees of freedom; T = time; F = iron; C = cobalt; subscript L = linear effect; subscript Q = quadratic effect.

Values of F at the 1% level = 7.88 (1 D of F) and 4.76 (3 D of F).

SOURCE OF VARIATION	D OF F	OBSERVATIONAL SET				
		Dry wt.	Protein	Total cobalt [1]	Soluble stable cobalt [1]	Particulate stable cobalt [1]
T	3	75	10.0			
F_L	1	303		10		16
F_Q	1	79			62	20
C_L	1		15	40	480	221
C_Q	1	15		9.7	97	38
$F_L \times C_L$	1	14		12		19
$F_L \times C_Q$	1					
$F_Q \times C_L$	1				86	27
$F_Q \times C_Q$	1				24	
ERROR	24	.052	17.91	33,055	3,101	3,874

[1] Variances calculated from the data in terms μM Co/100 mg dry weight of mycelium.

Similar other more or less complex curves could be presented for each experimental parameter and observational set. Inspection of such complex curves, indicating a multiplicity of interactions, is unlikely to reveal the basic factors involved. To gain this information, the analysis of variance was employed.

The details of the statistical analysis need not be presented here. The methods of Brandt ('49) and Snedecor ('46, pp. 253–373) were followed. The vector products for each of the

35 degrees of freedom for each of the 5 observables were calculated. Since this involved a 36 × 5 matrix multiplication, IBM punch cards were employed in the calculation.[2] Also using the IBM 604 Electronic Calculating Punch all the cross products and the fourteen 1 × 1 covariant sets were computed. The results of the statistical analysis pertinent to the question of iron-cobalt interaction are presented in tables 1 and 2. The values are for F, while blank spaces represent values not significant at the 1% level. The error terms are composed of all third order interactions, as well as those second order interactions shown not to be significant in a preliminary calculation. The homogeneity of the error variances was tested by Bartlett's test (Snedecor, '46, p. 250).

TABLE 2

Covariance analysis adjusted for dry weight

Entries and symbols same as table 1. NS indicates a change from significance to non-significance at the 1% level upon adjustment for dry weight.

SOURCE OF VARIATION	OBSERVATIONAL SET			
	Protein	Total cobalt	Stable soluble cobalt	Stable particulate cobalt
T	NS			
F_L		NS		NS
F_Q			NS	NS
C_L	NS	25	624	213
C_Q		NS	29	NS
$F_L \times C_L$		NS		NS
$F_L \times C_Q$				11
$F_Q \times C_L$			30	NS
$F_Q \times C_Q$			23	13
ERROR	17.15	34,425	1,934	3,236

Examination of the values of F, the relative mean variances, tabulated in table 1 disclose a considerable number of significant direct effects and interactions. One expected result is to find that cobalt accumulation in both the total and the metabolically incorporated stable fractions shows a high dependence on the cobalt concentration. However, the direct cobalt effect on growth was not anticipated, since attempts in the past to show a growth requirement for cobalt had been unsuccessful (Ballentine and Stephens, '51). It is also to be noted in this connection that there is a cobalt-iron interaction on growth. Further analysis of the data may provide the clue to a direct experimental demonstration of a partial growth-cobalt interdependence, and hence demonstrate at least the partial essentiality of cobalt for growth in *Neurospora*. Such specific experiments will be reported in a later communication.

---

[2] Thanks are due Mr. John Brown and Mr. Bruce Smith, of the Service Bureau, International Business Machines, Baltimore, and to Mr. Wade Etchisson, of the Computer Laboratory, The Johns Hopkins University.

Particular interest for the purpose of this report lies in the direct iron effects. Again, as would be predicted from data such as shown in figure 1, there is a highly significant direct iron effect on cobalt uptake. However, the relative variances of these effects are of a different order of magnitude from the direct effect of iron on growth itself. This suggests that iron may well be influencing cobalt accumulation *indirectly* through its influence on growth. Therefore, the covariance analysis presented in table 2 was made in which the variances are adjusted for dry weight. When this is done, all the *direct* iron effects on cobalt accumulation vanish. On the basis of this analysis iron and cobalt cannot be directly substituting for each other.

The amount of growth may be considered as an integrated growth rate. Similarly the specific activity, µM Co/100 mg dry weight, is the ratio of an integrated growth rate and an integrated rate of cobalt accumulation. Therefore, if the above deduction is correct, that iron indirectly affects cobalt uptake through its influence on growth, then the *rate* of growth should be directly correlated with the *rate* of cobalt accumulation and independent of the iron concentration.

In order to use the data at all cobalt concentrations, it is necessary to eliminate the *scale factor* or specific concentration term. This most readily can be accomplished without distortion of the data by reduction to *standard measure* (Thurstone, '47, pp. 63–67):

$$\hat{y} = \frac{Y - \bar{y}}{\sigma} \qquad (1)$$

where

Y = observed value
$\bar{y}$ = mean of the set of observed values
σ = their standard deviation
$\hat{y}$ = observed in standard measure.

and

The plot in figure 2 is for all concentrations of cobalt and iron where the rate of total cobalt uptake is given in standard measure. These rates, as well as the rates of growth, were derived from the data by making the considerable as-

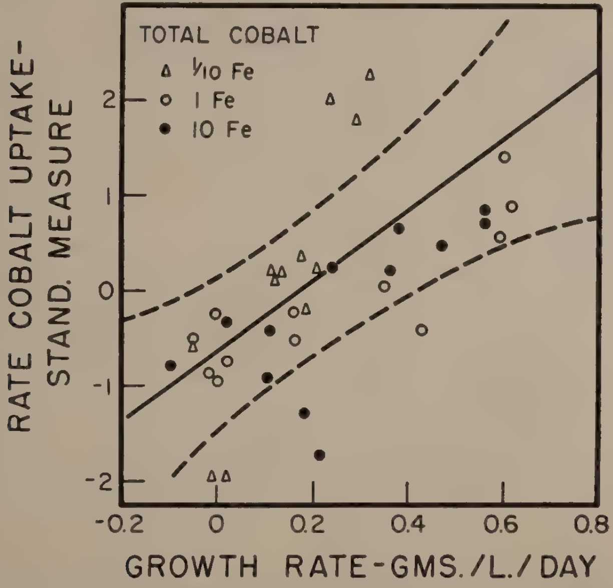


Fig. 2 Rate of growth of Neurospora and rate of cobalt accumulation.

Data for all cobalt concentrations at 1/10 Fe (△), 1 × Fe (○), and 10 × Fe (●). The solid line is calculated from $\hat{y} = 3.11\,X - 0.62$, the least squares fit. The dashed lines enclose the 95% confidence band.

Ordinate: Rates of total cobalt accumulation in units of standard measure ($\hat{y}$) per day.

Abscissa: Rate of growth in units of gm dry weight/L/day. The negative values occur late in the growth of the culture when some autolysis is taking place (10–14 days).

sumption that the rates were linear between times of measurement. This assumption alone could account for the large amount of scatter shown. However, the least squares fit was computed for the case where both X and Y are subject to error by the solution of the normal equations given in Sect.

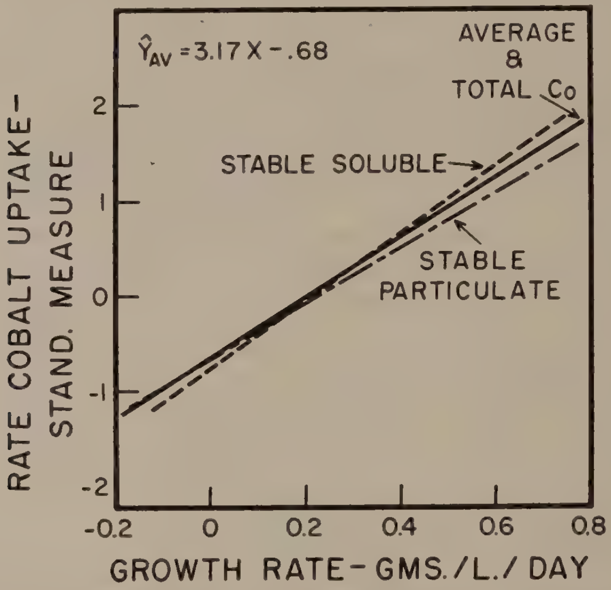


Fig. 3 Least squares curves relating rate of growth to total, stable-soluble and stable-particulate cobalt accumulation.

The solid curve, $\hat{y} = 3.17\ X - 0.68$, is the average for all three observational sets comprising all cobalt and iron concentrations.

Ordinate: Rate of cobalt accumulation in units of standard measure per day.

Abscissa: Rate of growth in units of gm dry weight/L/day.

60 by Deming ('43). The solid line in figure 2 is this fitted curve, $y = 3.11\ X - 0.62$, for total cobalt accumulation. The dashed lines enclose the 95% confidence band.

When a similar rate plot for soluble and particulate stably-bound cobalt is made and fitted by least squares, the curves shown in figure 3 are obtained. There is no significant difference between their slopes and intercepts, and the data for all concentrations of iron and cobalt for all forms of accumulated cobalt may be given by

$$\hat{y} = 3.17\,X - 0.62 \qquad (2)$$

where $\hat{y}$ is the rate of cobalt uptake in standard measure and x is the rate of growth in gm/L/day.

This treatment of the data provides a confirmation of the results of the covariance analysis that iron and cobalt metabolism is only *indirectly* related through a general growth effect. In addition it reveals that in these experiments the main metabolic factors controlling cobalt accumulation are common for all three cobalt components.

TABLE 3

Parameters for calculation of relation between rates of growth and rates of cobalt accumulation in actual concentrations

$y = a(x) \text{-} b$

where y = rate of cobalt uptake, μM/L/day, and x = rate of growth, gm dry weight/L/day

OBSERVABLE	COBALT CONCENTRATION	MEAN $\bar{y}$	STAND. DEV.	a μM/L/day	b μM/L/day
Total cobalt	1 ×	1.23	3.52	11.1×10^{-4}	1.16×10^{-4}
	10 ×	.552	1.65	5.23×10^{-3}	$.57 \times 10^{-3}$
	100 ×	.43	2.31	7.32×10^{-2}	1.14×10^{-2}
Soluble stable cobalt	1 ×	.221	.370	1.17×10^{-4}	$.03 \times 10^{-4}$
	10 ×	1.67	1.72	5.45×10^{-4}	-0.50×10^{-4}
	100 ×	.787	1.28	4.06×10^{-3}	$.08 \times 10^{-3}$
Particulate stable cobalt	1 ×	.174	.408	1.29×10^{-4}	$.103 \times 10^{-4}$
	10 ×	1.27	1.33	4.22×10^{-4}	$-.36 \times 10^{-4}$
	100 ×	.89	.91	2.89×10^{-3}	$-.26 \times 10^{-3}$

Combination of equations (1) and (2), together with the values for the means and standard deviations, allows the reconversion from standard measure back to the fitted data in terms of actual concentrations. Table 3 provides this information, and from these values together with the growth curves presented in figure 1, one may readily calculate the fitted rate and integral growth curves for cobalt accumulation.

In conclusion we may sum up the basic effects factored out of the complex growth data by converting both rates of growth and rates of cobalt accumulation to standard measure. This gives $\hat{y} = 0.66\,\hat{x}$. The slope of 0.66 means that the faster the mold increases in mass the *relatively* poorer in cobalt it will be. Slow growth rates, therefore, increase the cobalt *concentration;* in other words, for any given final mass of mycelium, the slower it attains this mass the more cobalt it will finally contain. In the case of iron, this relationship accounts for the observed high specific activity found at low iron concentrations where the rate of growth is severely depressed from the normal.

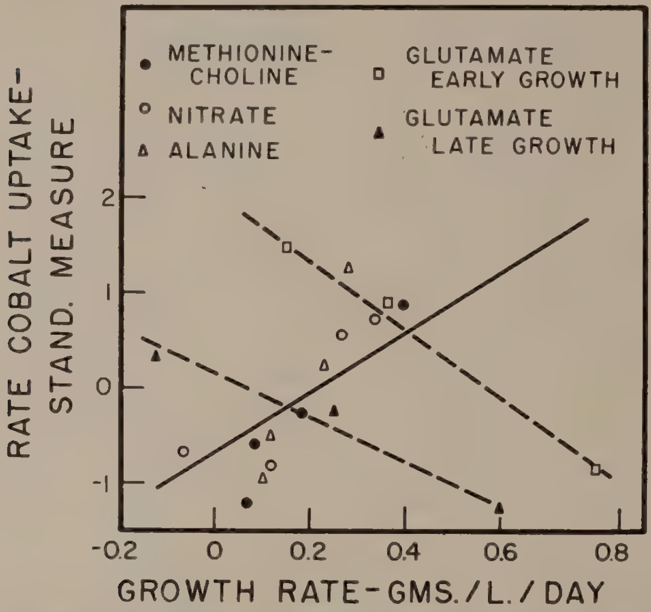


Fig. 4 Effect of other growth controlling factors on the relationship between rate of growth and rate of total cobalt accumulation.

The solid line is calculated from $\hat{y} = 3.17\,X - 0.68$.

The nitrogen sources are all present at the level of 25 mM/L of medium. The methionine (250 mg/L) and choline (25 mg/L) are added to the regular ammonium acetate medium.

Ordinate and abscissa as for figure 2.

The iron concentration, together with the physiological age of the culture, determines the rate of growth under the conditions in the experiments so far discussed. This rate in turn defines the rate of cobalt accumulation, according to Eqn. (2). It is of interest to examine the generality of this relationship for other experimental conditions. Both the growth rate and the total level of growth may be altered by changing the nitrogen source or by inhibition, such as by the addition of methionine plus choline to the medium. In figure 4, data for several experiments of this type are plotted as in figure 2. The curve is the least squares fit for the iron-cobalt data. Nitrate, alanine, and methionine-choline all fit this curve reasonably well considering that a different wild type strain (5297A) of *Neurospora* was used. Glutamate provides a striking exception. This growth behavior on glutamate will be the subject of a subsequent publication. The curves are included here to show that this type of analysis may provide a convenient test for separating some of the common indirect actions of experimental variables from more direct effects.

SUMMARY

1. An experiment of factorial design is presented to test the possibility of a metabolic mistake incorporating cobalt in place of iron into the mycelium of *Neurospora*.

2. Analyses of variance and covariance show that all direct iron effect on cobalt accumulation vanishes when the variances are adjusted for growth. Therefore, cobalt is not directly substituted for iron.

3. A direct cobalt effect on growth, and a cobalt-iron interaction on growth was detected. This suggests reexamination of the essentiality of cobalt for growth.

4. The equation $\hat{y} = 3.17\,X - .68$ relates the rate of growth in gm/L/day to the rate of cobalt accumulation in standard measure. This relationship holds for total, stable soluble, and stable particulate cobalt at all concentrations of cobalt and iron tested.

5. A table of parameters is presented allowing the calculation of fitted curves for cobalt accumulation in terms of concentration.

6 The slower the mold reaches a given growth level the higher the cobalt concentration, so that depressing the growth rate increases the specific cobalt activity.

7. The relationship of (4) is shown to fit some other growth depressing and stimulating factors, and may provide a test for separating the indirect action of experimental variables from the more direct effects.

It is doubtful if the work described in this paper could have reached completion without the able assistance of Dorothy Stephens de la Haba and Dorothy Burford. In addition to expressing my thanks to them, I wish to also acknowledge the many valuable critical discussions of this material so kindly provided by Dr. A. E. Brandt.

LITERATURE CITED

BALLENTINE, R., AND D. G. STEPHENS 1951 The biosynthesis of stable cobalto-proteins by plants. J. Cell. and Comp. Physiol., *37:* 369–387.

BRANDT, A. E. 1949 Forms of Analysis for Either Measurement or Enumeration Data Amenable to Machine Methods. Proc. Computation Seminar, IBM Corp., New York, pp. 149–153.

DEMING, W. E. 1943 ''Statistical Adjustment of Data,'' New York City.

SNEDECOR, G. W. 1946 ''Statistical Methods,'' Fourth Ed., Ames, Iowa.

THURSTONE, L. L. 1947 ''Multiple-Factor Analysis,'' Chicago, Illinois.

MUSCLE MEMBRANE POTENTIAL, RESISTANCE, AND EXTERNAL POTASSIUM CHLORIDE

HOWARD P. JENERICK
Department of Biology, Massachusetts Institute of Technology, Cambridge

SIX FIGURES

The measurement of electrical properties of cells contributes to a basic understanding of membrane function. One indisputedly important activity of the cell membrane is the control of solute and solvent penetration, manifested in the familiar concept of permeability. An equally important concept for ionic penetration is the electrical resistance of the membrane, because moving ions constitute an electric current. Analysis of this cellular electrical property is complicated by the varied internal and external ion environments and the likelihood that the membrane has several separate mechanisms for ion movement, active and passive. Nevertheless, it would be useful for better understanding of mechanism and also for describing measurable properties of the cell to measure the resistance of the membrane and compare this to the predictions of a membrane model. Potassium and chloride appear to be distributed according to their electrochemical gradient (Ussing, '49), and a study of their effect on membrane potential and resistance should not be complicated by the presently unassessable factors of active transport. Furthermore, the equations of Goldman ('43), derived for a membrane model and physical driving forces will be assumed valid and can be used to test the results. The present study was directed at the problem essentially as outlined above. The complex ionic movements for short times after a polarizing current is passed have not been studied.

METHODS

The isolated sartorius muscle of *Rana pipiens* was placed in a small chamber, through which flowed the experimental solutions. Two glass capillary microelectrodes, filled with 3 M KCl, were inserted into one muscle fiber within 100 μ of each other. The bath was connected to ground by a Ringer bridge and calomel cell, and the microelectrodes were connected through saturated KCl bridges and calomel cells to the other equipment. One of the microelectrodes recorded potential changes and was connected to a d.c. cathode follower which led through a potentiometer to the vertical d.c. amplifier of an oscilloscope. When this electrode pierced the cell, the oscilloscope spot was deflected by the transmembrane potential. With the potentiometer the spot was returned to the original position, about which it now responded to changes in resting potential due to polarization. The potentiometer reading, corrected for the cathode follower gain, gave a reading of the resting potential. The polarizing electrode led from a variable current source. Polarizing current was monitored with a series resistor and the oscilloscope's horizontal d.c. amplifier. Vertical oscilloscope deflections were proportional to potential changes and horizontal deflections proportional to polarizing current (resembling the arrangement of Cole and Curtis, '41). The current source was varied so that the membrane potential was steadily raised or lowered by 20–80 mV. in 5 to 10 seconds. As the spot moved across the oscilloscope tube face, tracing out the curve of potential as a function of current, a camera recorded its path. Known currents and voltages were applied for calibration. After a series of polarization curves were recorded for fibers in normal Ringer's solution (111.0 mM. NaCl, 2.5 mM. KCl, 1.3 mM. $CaCl_2$, 2.4 mM. $NaHCO_3$, 1.0 mM. phosphate buffer at pH 7.3) the test solution was then run through. The surface fibers were allowed to equilibrate with the new fluid for 5 to 15 minutes before any readings were made. In all experiments no marked changes were seen in the hour or less following the change.

CALCULATION OF MEMBRANE RESISTANCE

The experimental method does not measure membrane resistance directly. The current passing through the membrane falls off with distance from the electrode proportional to the membrane resistance for small currents. The ratio between membrane potential change and total polarizing current has the dimensions of a resistance, but it is a function of electrode separation and membrane properties and must be corrected as follows.

Definitions

V = change in membrane potential at x due to polarizing current (volts).
X = distance along fiber, polarizing electrode at $x = 0$ (cm.).
V_o = change in membrane potential at $x = 0$ due to polarizing current (volts).
r_m = resistance of a unit length of membrane, equals $R_m/2\pi a$ (ohm cm.).
r_e = resistance of unit length of external Ringer's solution (ohm/cm.).
r_i = resistance of unit length of myoplasm, equals $R_i/\pi a^2$ (ohm/cm.).
R_m = specific resistance of membrane (ohm cm.^{2}).
R_i = specific resistance of myoplasm (ohm cm.).
a = radius of muscle fiber (cm.).
i_m = current penetrating membrane at x (amperes).
I_o = total current flowing through membrane (amperes).
λ = space constant = $\sqrt{r_m/(r_i + r_e)}$ (cm.).

The muscle fiber will be considered a cable conductor with an internal polarizing electrode located at $x = 0$. Steady state conditions only will be discussed. From the definitions as given and assuming the membrane resistance remains constant during current flow, it follows that,

$$V = V_o\, e^{-|x|/\lambda} \qquad (1)$$

By Ohm's law,

$$V = r_m i_m$$

therefore,

$$i_m = \frac{V_o}{r_m}\, e^{-|x|/\lambda}$$

by definition,

$$I_o = \int_{-\infty}^{+\infty} i_m dx = 2 \int_{0}^{+\infty} \frac{V_o}{r_m} (e^{-x/\lambda})\, dx$$

since the current is symmetrical about $x = 0$.

Integrating between limits,

$$I_o = \frac{2V_o}{r_m} \cdot \lambda$$

Substituting for λ, clearing, and rearranging,

$$r_m = \left(\frac{V_o}{I_o}\right)^2 \cdot \frac{4}{r_i + r_e} \qquad (2)$$

an expression is obtained relating the resistance of a unit length of membrane to the fixed resistances, r_i and r_e, and the experimental data, V_o and I_o. In general, r_i is much larger than r_e and the latter term may be dropped from the denominator. The internal resistance will be considered constant, since there is no information available on its possible variation in altered Ringer's solutions, and internal ion concentrations are relatively independent of external ion changes for short times. R_i was assumed to be 250 ohm cm.

Solving for the specific membrane resistance,

$$R_m = \left(\frac{V_o}{I_o}\right)^2 \cdot \frac{8}{(R_i/\pi^2 a^3)} . \qquad (3)$$

Because of the necessary separation of polarizing and recording electrodes, the oscilloscope traces V as a function of I_o. V is then corrected to V_o as follows. A trial r_m was calculated from the experimental value for V/I_o. The space constant was calculated from the trial r_m and this was used in (1) to correct V to V_o. The value of V_o/I_o then gave a new r_m and λ which were used to recorrect the original V. Repeating this process several times finally gave the r_m and R_m values reported here. For small I_o, V_o is a straight line function of I_o, and the slope of its curve through the origin is taken as the total membrane resistance R (Cole and Curtis, '41; Katz, '48). A maximum error of 5% probably occurs in tracing the curves from film and estimating slopes. The term V_o/I_o, however, enters all calculations of r_m and R_m as a square and this error is raised to 10%. A long working distance is required of the microscope objectives, and optical magnification is limited, but repeated measurements of diameter on one fiber agree within 5%. Unfortunately, the radius enters equation (3) as the cube, and the 5% radius uncertainty is magnified into 15% for R_m. A third source of error, and one that cannot be properly assessed, is that the equations are derived for a single, isolated fiber, whereas in practice the fiber tested is surrounded on three sides by adjacent fibers. $\bar{R}$ in table 1 was not corrected.

Polarizing currents alter membrane resistance, and therefore electrotonic spread cannot be exponential. Equations (2) and (3) are valid for exponential decay only. Curve slopes not at the origin cannot be used. However, if it is

TABLE 1

EXPERIMENT	TEMPERATURE	$\bar{R}$ (ohms × 10^5)	DIAMETER	MEMBRANE POTENTIAL
	°C.		μ	mV.
19	25.0	4.00	103	79
		3.10	103	76
		3.10	113	74
17	26.5	4.10	95	80
		3.22	100	81
		3.83	95	79
13	28.0	2.47	80	88
		2.74	100	84
		3.20	90	80
		2.30	70	86
11	27.5	2.44	90	83
		2.71	80	84
		2.44	90	83
		3.04	70	85
		2.62	100	83
		2.28	80	87
4	25.0	2.40	80	80
		3.50	80	84
		3.30	85	80
3	23.0	2.70	90	80
		2.80	90	81
		3.10	90	79
		2.30	95	78
2	...	3.22	89	83
		4.40	89	82
		3.57	85	84
		3.21	98	87
		2.90	107	81
		2.96	80	81
		3.20	94	81
Averages		3.04 ± 0.55	90.4 ± 11	82.2 ± 3.2

assumed that the non-linearity of voltage and current is monotonic, that is to say, the voltage-current curve has no maxima or minima (and it does have none in the experimental range) then an approximation can be made. The slope at any point and equations (2) and (3) will give resistances showing less non-linearity with current than is really present, but never more, and one will underestimate the resistance change due to polarization but never overestimate. Within this limitation, it is possible to calculate the membrane resistance changes as a function of polarizing current.

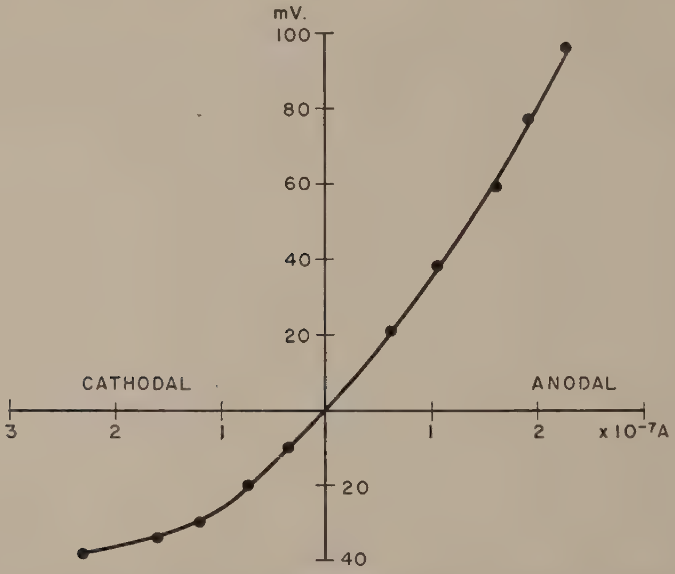


Fig. 1 Changes in resting transmembrane potential of single muscle fiber induced by polarizing current. Resting potential = 82 mV. Membrane specific resistance = 2250 ohm cm².

RESULTS

Resistance in normal Ringer's solution

Resistance measurements were made on a total of 30 fibers in 2.5 mM. K^+ Ringer's fluid to obtain the value of the normal resistance. Table 1 presents these data. R_m, calculated by equation (3) from the averages of $\bar{R}$ and a, is 2500 ± 800 ohm cm². Figure 1 presents the polarization curve of one such fiber. The tangent at the origin corresponds to a R of 3.05×10^5 ohms. This fiber's diameter was 82 μ; therefore, R_m was 2250 ohm cm². The specific resistance rose to at least 7600 ohm cm² anodally and fell to at least 160 ohm cm² cathodally, illustrating appreciable rectification.

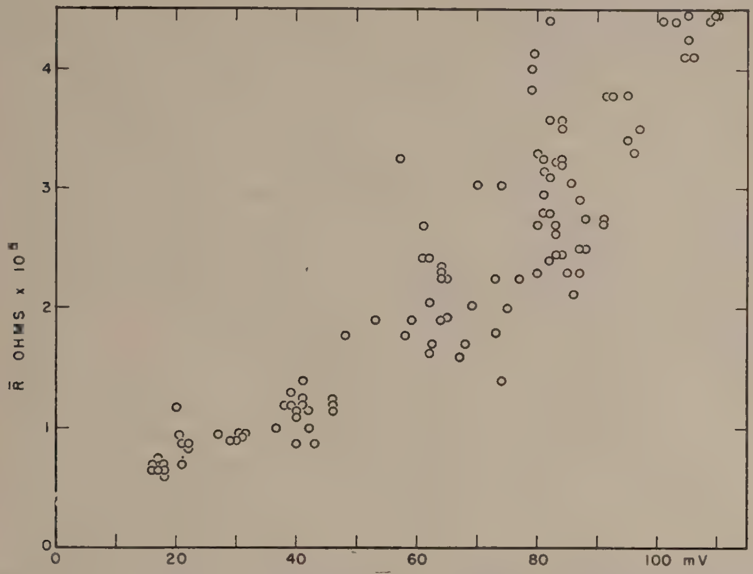


Fig. 2 Total membrane resistance, $\bar{R}$, of individual single fibers in varying KCl-Ringer's solutions plotted against their membrane potential. $\bar{R}$ = slope of polarization curves at zero current.

Rectification

With reference to figure 1 and the calculated resistance changes, this fiber exhibited the normal type of rectification. No quantitative measures were made of this phenomena, but in all experiments it was noted whether the curve flattened, corresponding to less rectification, or became concave downwards, indicating a reversal. Summing up these qualitative observations: increasing K^+ concentrations tend to flatten the curve but not reverse it, with a fairly straight line over the range ± 20 mV. appearing above 7.5 mM. K^+. If the 1.0 mM. phosphate buffer was omitted from the test solution the rectification reversed and the resistance then decreased anodally and increased cathodally. The rectification decrease with K^+ has already been reported (Guttman, '44).

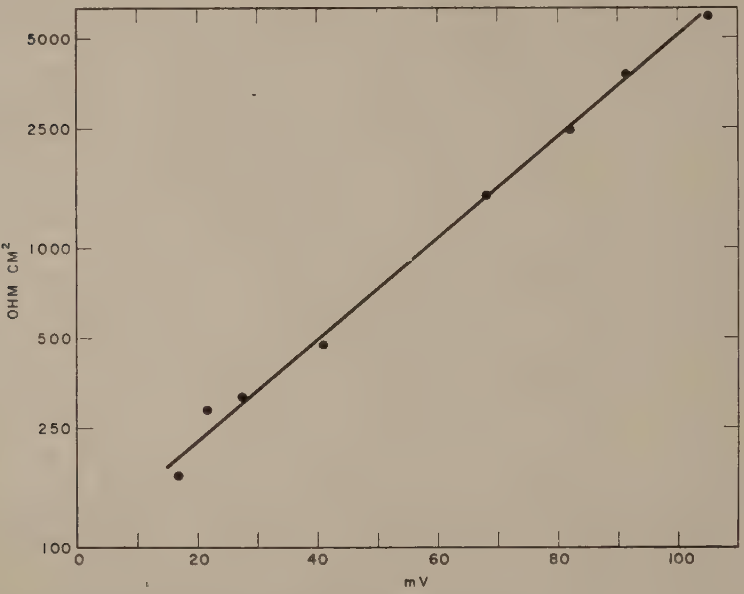


Fig. 3 Log of membrane specific resistance averages in different KCl-Ringer's solutions plotted against average membrane potentials.

Resistance and potassium chloride concentration

Solid KCl was added to the stock Ringer's solution in concentrations from 0 to 110 mM. No provision was made for the altered osmotic pressures, only one parameter being desired. The osmotic effect could not have been too large since the fiber diameters remained fairly constant over the experimental range.

Figure 2 represents a plot of $\overline{R}$ against membrane potential for 105 fibers, the total number examined. Scattering is apparent but the trend obvious, lowered resistance accompanies lowered potential. Probably the three variables, KCl concentration, potential, and resistance, are interrelated in some fashion. For purposes of illustration only, potential was chosen as the independent variable with the justification that (1) rectification lowers resistance in electrically depolarized fibers, and (2) in a series of fibers in any given KCl concentration those with lower potential tend to have a lower resistance.

TABLE 2

$(K^+)_o$ mM./L	E mV.	$\overline{R}$ 10^5 ohm	r_m 10^4 ohm cm	R_m ohm cm²	NUMBER OF FIBERS
0.0	105	4.9	22.00	6000	8
1.0	91	3.9	14.00	3800	6
2.5	82	3.2	9.30	2500	32
7.5	68	2.5	5.60	1500	20
26.0	41	1.4	1.80	480	16
50.0	28	1.1	1.20	320	6
75.0	22	1.1	1.10	290	6
110.0	17	0.8	0.64	170	7

Average diameter = 86 μ.

The data of figure 2 were broken down into groups depending upon K^+ concentration, and these groups form table 2 and figure 3 where it is to be noted that log R_m is linear with E. This resistance-potential relationship occurs in squid giant axon (Hodgkin and Huxley, '52a) where log conductance is linear with E for small changes in resting potential caused by polarization. To forestall any confusion, the squid data were obtained with potential altered by polarization; the present muscle data were obtained with potentials altered to various new resting levels with KCl. Figure 2 was also broken down into 5 mV. potential groups, and the associated resistance values were averaged. This gave a linear relation very similar to figure 3.

Potassium permeability coefficient

Resistance, potential, and ionic concentrations are all interrelated by the following equations taken from Hodgkin and Katz ('49), based on Goldman's constant field theory ('43):

$$E = \frac{RT}{F} \ln \frac{y}{w} \qquad (4)$$

$$G = \frac{F^3}{(RT)^2} E P_K \left(\frac{yw}{y-w}\right) \qquad (5)$$

E = resting membrane potential (volts).
R, T, F = gas constant, absolute temperature, and Faraday.
G = conductance of membrane ($\text{ohm}^{-1}\text{cm}^{-2}$) also is $1/R_m$
$y = (K^+)_{in} + P_{Na}/P_K (Na^+)_{in} + P_{Cl}/P_K (Cl^-)_{out}$
$w = (K^+)_{out} + P_{Na}/P_K (Na^+)_{out} + P_{Cl}/P_K (Cl^-)_{in}$
P_K, P_{Na}, P_{Cl} = permeability coefficients for K^+, Na^+, and Cl^-, respectively.

Solve (4) for w and substitute into (5).

$$G = B P_K E \frac{y\,(10^{-E/.059})}{(1 - 10^{-E/.059})}$$

where $B = F^3/(RT)^2$. Recalling that $G = 1/R_m$, one can write:

$$10^{E/.059} - 1 = B E P_K y R_m \qquad (6)$$

which determines P_K in terms of the experimental variables E and R_m, the constant B, and y. Values for y are estimated from the potential studies reported later.

If E and P_K are inversely proportional to each other, their product will be a constant, and equation (6) can be reduced to a simple linear equation. Experimental values for $(10^{E/.059} - 1)$ are plotted as a function of y R_m in figure 4, where the linearity implies that B E P_K is constant. It seems, therefore, that the permeability coefficient of K^+, P_K, is inversely proportional to the resting potential from 17 to 91 mV. For an E of 82 mV. P_K is 5×10^{-6} cm/sec.

Membrane potential

In its complete form, equation (4) is:

$$E = .059 \log \frac{(K^+)_i + \frac{P_{Na}}{P_K}(Na^+)_i + \frac{P_{Cl}}{P_K}(Cl^-)_o}{(K^+)_o + \frac{P_{Na}}{P_K}(Na^+)_o + \frac{P_{Cl}}{P_K}(Cl^-)_i} \qquad (4)$$

This specifies the resting potential with 6 ion concentrations and two constants. The two constants were determined as follows: internal ion concentrations were taken from Boyle and Conway ('41) and the external ion concentrations and membrane potentials were taken from experiments at 1.0 and 7.5 mM. K^+, giving two simultaneous equations with two unknowns, the permeability ratios. Calculated in this

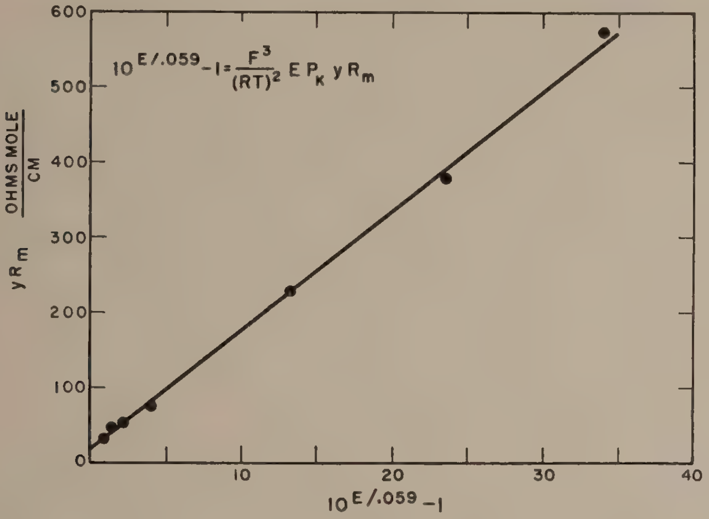


Fig. 4 Graphical test of equation (6). Points indicate experimental values, straight line indicates linearity of data. Ordinate to be multiplied by 10^{-3}. See text.

TABLE 3

$[K^+]_o$	$[Cl^-]_o$	$[Na^+]_o$	^{E}Calc.	^{E}EXPERIMENT
1.0	115	113	91	91
2.5	116	113	83	82
7.5	121	113	68	68
26.0	140	113	43	41
50.0	164	113	29	28
75.0	189	113	20	22
110.0	224	113	12	17

Internal ion concentrations, $[K^+]_i = 125$, $[Na^+]_i = 15$, $[Cl^-]_i = 1.2$, taken from Boyle and Conway ('41). Concentrations expressed as millimoles per kilogram water in fiber or solution. Potentials expressed in millivolts and rounded off to two significant figures.

manner, $P_{Na}/P_K = 0.027$ and $P_{Cl}/P_K = 0.23$. These values along with experimental K^+, Na^+, and Cl^- concentrations were used in (4) to predict the membrane potential. Table 3 presents the predicted and measured potentials. Figure 5 illustrates the close agreement. One unexplained discrepancy was found. Membrane potentials in zero K^+ were approximately 105 mV., whereas the calculated potential for this concentration is only 98 mV. The values of y needed for a previous section were calculated from these permeability ratios.

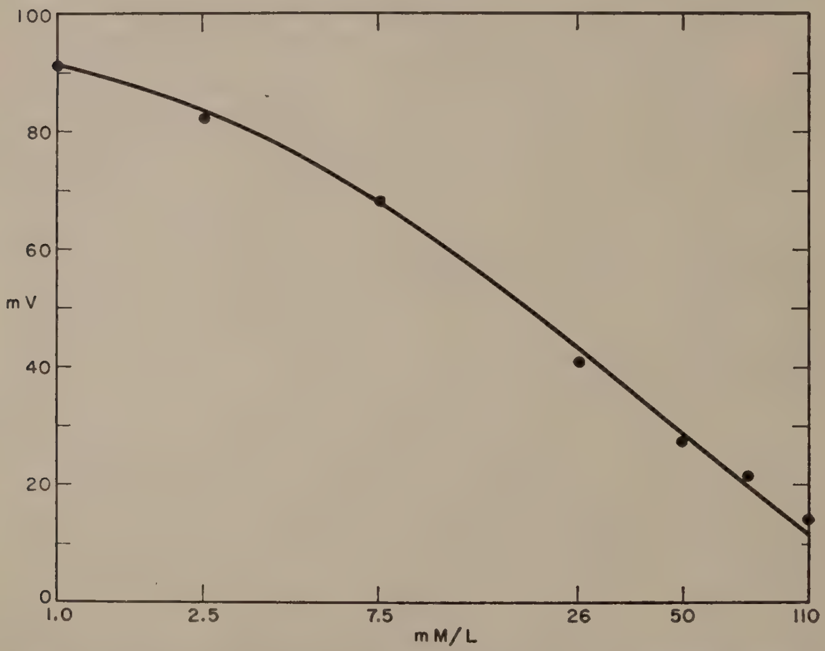


Fig. 5 Membrane potential and KCl concentration of experimental Ringer's solutions. Open circles indicate potentials measured with an intracellular electrode, smooth line is predicted from equation (4) with $P_{Na}/P_K = 0.027$ and $P_{Cl}/P_K = 0.23$.

Space constant

Purely as a matter of interest and because the relevant data were at hand, the space constant was calculated from the various r_m values obtained. This is presented in table 4. Figure 6 plots the space constant against the membrane potential and illustrates the nearly 10-fold change which occurs.

TABLE 4

E	[K^+]	SPACE CONSTANT
mV.	*mM.*	*mm*
105	0.0	2.40
91	1.0	1.91
82	2.5	1.54
68	7.5	1.20
41	26.0	0.68
28	50.0	0.54
22	75.0	0.52
17	110.0	0.41

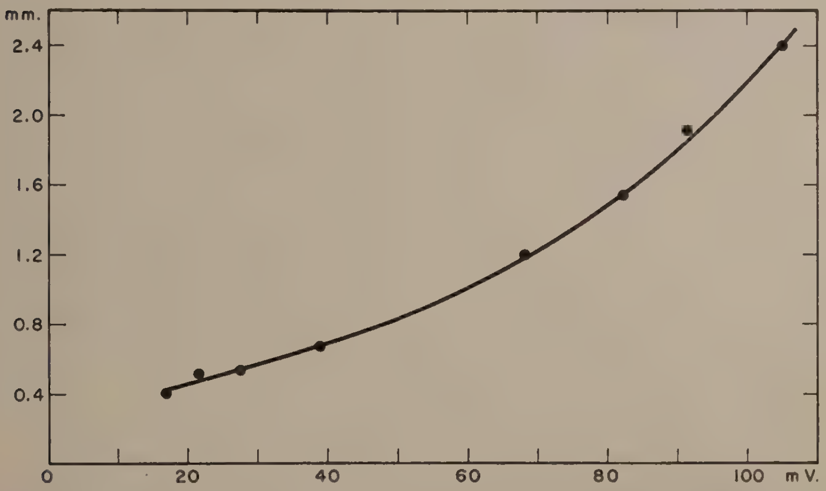


Fig. 6 Variation of space constant, λ, with resting potentials altered by KCl in Ringer's solution.

DISCUSSION

It is necessary to make explicit that the experiments were not designed to distinguish between currents carried by K^+ and other ions. The experimental solutions were altered in K^+ and Cl^- content simultaneously. However, resting fluxes of tracer K^+ and Cl^- seem to be about equal in frog sartorius

(Hodgkin, '51). In squid giant axon, for potentials near the resting value, about one-half of the membrane current is carried by K^+. The other one-half to a large extent is perhaps carried by Cl^- (Hodgkin and Huxley, '52b). Consequently it seems probable that K^+ and Cl^- are equally and mainly responsible for the conductances measured in the present work.

The specific membrane resistance of frog sartorius fibers in 2.5 mM. K^+ Ringer's solution is 2500 ohm cm². Previously reported values obtained with other experimental techniques have been: 40 ohm cm² (Bozler and Cole, '35), 2100 ohm cm² (Katz, '48), and 4100 ohm cm² in 2.0 mM. K^+ (Fatt and Katz, '51). The low value of Bozler and Cole may be due to their procedure which entailed placing the muscle on the electrodes in air for upwards of one hour. Muscle is very liable to leak K^+ under these conditions and depolarize itself. The increased external K^+ concentration or the depolarization can cause a large reduction in resistance.

The larger figure reported by Fatt and Katz is partially due to the lower K^+ concentration used (the value for 2.0 mM. K^+ in the present work would be 2900 ohm cm²), and may also be partially due to their method of calculation. They measured the potential, V, about a polarizing electrode and solved simultaneously the following expression for r_m and r_i after substituting experimental values of V for two different electrode separations, *x*:

$$V = \frac{I}{2}(r_m r_i)^{\frac{1}{2}}\, e^{-x(r_i/r_m)^{\frac{1}{2}}}$$

Solving this for r_i:

$$r_i = \frac{1}{2}\left[\frac{4}{r_m I^2}\left(\frac{V_1{}^{x_2}}{V_2{}^{x_1}}\right)^{2/x_2-x_1} + r_m\frac{(\ln\frac{V_1}{V_2})^2}{(x_2-x_1)^2}\right]$$

Experimentally they used anodal polarizations which increases r_m and λ near the electrode because of rectification which is present in 2.0 mM. K^+ solutions. The ratio V_1/V_2 decreases from what it would be if no rectification occurred.

This alteration causes either an underestimation of r_i or an overestimation of r_m. The fiber diameter d was then calculated from r_i.

$$d = \sqrt{\frac{4}{\pi} \cdot \frac{R_i}{r_i}}$$

Since the calculated value for r_i may have been lowered because of rectification, d will be overestimated. This may account for the large diameter values they calculated, a 140 μ average. As was pointed out in a previous section, d enters R_m calculations as a cubed term, and R_m will be overestimated if this occurs. If the other possibility is true, i.e., r_m overestimated, this too will increase R_m. Having no idea of the actual departure of the V-x curve from the theoretical curve, one cannot estimate how much of a difference this causes. It may be negligible, it may not.

A legitimate question that may be asked of the present work is: do polarizing currents of several seconds' duration alter the resistance in some fashion differently from the physiological currents of a few milliseconds? This seems unlikely because potassium conductances in lobster nerve remain at a steady value after a few milliseconds to over 300 milliseconds (Hodgkin and Rushton, '46).

Rectification reverses in sartorius with isotonic solutions of K_2SO_4 and sucrose (Katz, '48). The finding, reported briefly in the present work, that phosphate lack reverses rectification may be relevant. It cannot be decided *a priori* whether phosphate lack is the only way to reverse rectification, nor can it yet be decided, *a posteriori,* just how the absence of phosphate causes the reversal. A change in pH near the electrode may be the answer, especially if the membrane components are far from their isoelectric point and have a weak buffer capacity. Another possibility is this — phosphate ion is present in frog plasma at about 3 mM. It has also been localized, by radioautograph, in or near the surface of the cell from which it is easily washed away in zero phosphate solutions (Causey and Harris, '51). If the ion is really in equilibrium between the membrane and the external milieu, it appears probable that removal of solution phosphate should deplete membrane phosphate and change the surface structure. A most pertinent observation is that of Fenn and Cobb ('34). Measuring the leakage of K^+ from frog muscle, they found that 0.006 M phosphate lowered the outside K^+ concentration needed to stop the leakage. This illustrates a dependence of some sort between transmembrane K^+ movement and phosphate ion.

The decrease in membrane resistance with K^+ is not unique for frog sartorius; it is also found in crab nerve (Hodgkin, '47b) and in squid giant axon (Cole, '49). Rather puzzling in this regard is the report by Guttman ('38) that isotonic KCl produces only a ½% change in the one kilocycle impedance of frog sartorius. Solutions were dripped onto filter paper strips serving as electrodes on both sides of the muscle. Possibly the two strips touched and short-circuited the muscle. Indicative of this possibility is the author's statement that the impedance change occurred immediately after new solutions were applied to the strips. Diffusion into whole muscle usually does not occur this rapidly.

A reasonably valid assumption is that K^+ moves through muscle membrane under the driving force of its electrochemical gradient, since no evidence of active transport has been discovered (Ussing, '49). The equations used here are based upon such a driving force. Substitution of the experimental data in these equations led to the prediction that the K^+ permeability coefficient is an inverse function of membrane potential. Defined in basic terms:

$P_K = \mu_K B_K RT/tF$.
μ_K = mobility of K^+ ion.
B_K = partition coefficient of K^+ between membrane and surroundings.
R,T,F = gas constant, absolute temperature, and Faraday.
t = membrane thickness.

Therefore one or more of the three variables, μ_K, B_K, or t must be inversely proportional to E. If it is μ_K, then an interesting problem occurs. Ionic mobilities are defined for unit voltage gradient, so that mobility in aqueous solution cannot be a function of potential. However, membrane potentials were lowered by increasing KCl concentration in the Ringer's fluid. One possibility is that membrane structure is also dependent upon KCl concentration. Tobias ('52) has summarized evidence tending to show that K^+, in axoplasm at least, hydrates and disperses structure and increases permeability. If such a structural loosening and water uptake also occurs in the membrane then it would be expected that K^+ increases, which lower potential, would simultaneously make it easier for water soluble particles to enter. Indicative of this possibility is the deduction that P_{Na} and P_{Cl} also increase as the membrane potential is lowered, and the well-accepted fact that K^+ increases the permeability of other compounds (Heilbrunn, '43). A change in B_K would also be expected. P_K calculated from (6) is 5×10^{-6} cm sec.$^{-1}$, comparing favorably with estimates from tracer studies (Harris and Burn, '48).

Attempts are no longer made to interpret muscle membrane potential exclusively as a K^+ diffusion potential. The demonstrated penetration by Na^+ and Cl^- are well known. Equation (4) gives the membrane potential in terms of intra- and extracellular K^+, Na^+, and Cl^- concentrations and the relative permeability coefficient ratios P_{Na}/P_K and P_{Cl}/P_K. For squid giant axon, the ratios are $P_{Na}/P_K = 0.04$ and $P_{Cl}/P_K = 0.45$ in agreement with tracer studies (Hodgkin, '51). Comparable ratios of $P_{Na}/P_K = 0.027$ and $P_{Cl}/P_K = 0.23$ were obtained for frog sartorius (fig. 5). It must be mentioned that other, slightly different ratios can be calculated which more or less fit the data, but Hodgkin ('51) has pointed out that the apparent P_{Na}/P_K ratio for external ions, determined from tracer studies on sartorius is approximately 0.03 and for this reason the value of 0.027 was chosen. Since these ratios, assumed constant, were found to cause good agreement over the range 1 to 110 mM. K (excluding zero K^+ which did not) and a previous section noted that P_K rises as E falls, it follows that P_{Na} and P_{Cl} must, like P_K, be similarly dependent upon E. This lends credence to the previous suggestion that the membrane is non-specifically loosened by K^+. It may be a coincidence but the relative agreement of permeability ratios between squid axon and frog muscle may indicate a basic similarity of the two membranes. It would be useful from a comparative standpoint to extend this analysis to other phyla.

For the purpose of calculating the permeability ratios, it was assumed that the internal ion concentrations were not markedly altered by outside ion changes, despite Boyle and Conway's finding ('41) to the contrary. The justification for this is the short duration of these experiments, most of them about one hour; Boyle and Conway used two- to 24-hour periods. While not trying to rule out this variable, it has been assumed that the changes developed slowly enough during the one-hour experiment to be lost among the other experimental variables. Also, the potentials were averaged tending to reduce the variation caused by the gain or loss of intracellular ions. The potentials were observed to decline by perhaps several mV. toward the end of the hour, suggesting that ions were indeed entering.

These results bear on muscle threshold. Previous work (Jenerick and Gerard, '53) demonstrated that rheobase varies linearly with membrane potential in the range 68 to over 100 mV., both changing in the same direction. Below 68 mV. the rheobase increases, while below approximately 55 mV. propagated twitches disappear. The line of regression drawn through the experimental points in the 68 to 100 mV. range extrapolates to a zero-threshold intercept on the membrane potential axis at 57 mV. and it was postulated on this and other evidence that excitation occurs when the membrane potential is suddenly lowered to this level during stimulation. It was further predicted upon consideration of a simple membrane model that rheobase should be inversely proportional to membrane resistance and directly proportional to the potential fraction above 57 mV. A few preliminary measurements on membrane resistance at that time gave qualitative agreement to the theory. But now that more complete data are available on the variation of potential and resistance, it might be interesting to test the theory that there is an excitation potential of 57 mV. in muscle.

In order to calculate the depolarization at threshold, the membrane resistance $\bar{R}$ is multiplied by the strength of the threshold current; R being defined as the (potential change) /(current change). Since the resting conductance does not change appreciably during the *onset* of excitation (Hodgkin and Huxley, '52c, p. 529) this is a valid procedure. The product indicates the peak depolarization at the electrode but this depolarization decrements away in space more or less sharply depending upon the membrane resistance. If a minimum length of membrane needs to be depolarized, then the threshold will tend to rise at the small λ which are associated with lower membrane potentials. Since it was found that thresholds began to rise in the 57–68 mV. range, one explanation may be that there is a minimal length of membrane which must be brought to the critical potential. Another far more unassessable factor is the presence of the local response or "creep" seen at a cathode immediately following the start of current flow. Over and above what depolarization a threshold current will cause flowing through the electrical network of the membrane, this local response, present in muscle (Katz, '48), will add its bit. Only a detailed study of prepotentials with varying resting membrane potentials would indicate the real process. Such studies are planned.

TABLE 5

E	$I_{\text{STIMULATION}}$ 10^{-7} Amp.	$\bar{R}$ 10^5 ohm	IR	(E)−(IR)
mV.			mV.	mV.
90	1.15	3.9	45	45
82	1.00	3.2	32	50
68	0.60	2.5	15	53

Perhaps the best evidence yet found concerning this critical potential hypothesis is found in figure 27 of Fatt and Katz ('51). Applying polarizing currents during stimulation they recorded pre- and spike-potentials. Their records 2, 3, and 4 differ in resting potentials by about 10 mV. yet the action potentials and the *inflection point* (usually accepted as time of excitation) of the prepotentials are exactly superimposable. This indicates directly that it is not a critical *amount* of depolarization that excites, rather depolarization to a critical *potential.*

Purely as a corroboratory check of the critical potential hypothesis, table 5 was prepared from some unpublished data on rheobase and membrane potential and the resistances found associated with these potentials by the present work. Subtracting the IR drop across the membrane from the resting potential gives the potential which is assumed to exist at the moment of stimulation. Although the calculation neglects the effect of rectification, the results are at least consistent with the original hypothesis. No great insistence is placed upon these purely tentative calculations and they are merely presented as confirmation of the conclusions of Jenerick and Gerard ('53).

ACKNOWLEDGMENTS

The author is indebted to Dr. Martin Lubin and Dr. Robert E. Taylor for helpful discussions on some of the mathematical portions.

SUMMARY

Using penetrating microelectrodes, resting membrane potential, rectification, and resistance have been measured in single frog sartorius muscle fibers with altered KCl concentrations in Ringer's solution.

For permeability ratios of $P_K : P_{Na} : P_{Cl} :: 1 : 0.027 : 0.23$ and a modified diffusion potential equation (Hodgkin and Katz, '49) predicted membrane potentials agree well with measured potentials from 17 to 91 mV. The estimated value of P_K in normal Ringer's is $\sim 5 \times 10^{-6}$ cm sec.$^{-1}$. This permeability coefficient as well as those for Na^+ and Cl^- appears to be inversely proportional to membrane potential.

Rectification is reversed if phosphate ion is omitted from Ringer's and resistance increases at the cathode. It has been confirmed that raising outside K^+ concentrations decrease the rectifying properties of the membrane and tends to make it act as a simple ohmic resistance.

The specific membrane resistance in Ringer's with 2.5 mM. K^+ is 2500 ohm cm^2, rising to 6000 ohm cm^2 in zero K^+ Ringer's and falling to 170 ohm cm^2 in Ringer's with isotonic KCl. The log of membrane resistance is linear with membrane potential from 17 to 105 mV., resembling the behavior of squid giant axon membrane.

The space constant is 1.5 mm in normal Ringer's solution and may vary nearly 10-fold as membrane potential is changed by KCl.

LITERATURE CITED

BOYLE, P. J., AND E. J. CONWAY 1941 Potassium accumulation in muscle and associated changes. J. Physiol., *100:* 1–63.

BOZLER, E., AND K. S. COLE 1935 Electric impedance and phase angle of muscle in rigor. J. Cell. and Comp. Physiol., *6:* 229–241.

CAUSEY, G., AND E. J. HARRIS 1951 Uptake and loss of phosphate by frog muscle. Biochem. J., *49:* 176–182.

COLE, K. S. 1949 Dynamic electrical characteristics of the squid axon membrane. Arch. Sci. Physiol., *3:* 253–258.

COLE, K. S., AND H. J. CURTIS 1941 Membrane potential of the squid giant axon during current flow. J. Gen. Physiol., *24:* 551–563.

FATT, P., AND B. KATZ 1951 An analysis of the end-plate potential recorded with an intra-cellular electrode. J. Physiol., *115:* 320–370.

FENN, W. O., AND D. M. COBB 1934 The potassium equilibrium in muscle. J. Gen. Physiol., *17:* 629–656.

GOLDMAN, D. E. 1943 Potential, impedance, and rectification in membranes. J. Gen. Physiol., *27:* 37–60.

GUTTMAN, R. 1938 The electrical impedance of muscle during the action of narcotics and other agents. J. Gen. Physiol., *22:* 567–591.

——— 1944 Action of potassium and narcotics on rectification in nerve and muscle. J. Gen. Physiol., *28:* 43–51.

HARRIS, E. J., AND G. P. BURN 1948 Permeability of muscle cells. Nature, *162:* 929–930.

HEILBRUN, L. V. 1943 An Outline of General Physiology, 2nd ed. W. B. Saunders, Philadelphia, Pa.

HODGKIN, A. L. 1947 The effect of potassium on the surface membrane of an isolated axon. J. Physiol., *106:* 319–340.

——— 1951 The ionic basis of electrical activity in nerve and muscle. Biol. Rev., *26:* 339–409.

HODGKIN, A. L., AND A. F. HUXLEY 1952a Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. J. Physiol., *116:* 449–472.

——— 1952b The components of membrane conductance in the giant axon of *Loligo*. J. Physiol., *116:* 473–496.

——— 1952c A quantitative description of membrane current and its application to conduction and excitation in nerve. J. Physiol., *117:* 500–544.

HODGKIN, A. L., AND B. KATZ 1949 The effect of sodium ions on the electrical activity of the giant axon of the squid. J. Physiol., *108:* 37–77.

HODGKIN, A. L., AND W. A. H. RUSHTON 1946 The electrical constants of a crustacean nerve fiber. Proc. Roy. Soc. B., *133:* 444–479.

JENERICK, H. P., AND R. W. GERARD 1953 Membrane potential and threshold of single muscle fibers. J. Cell. and Comp. Physiol., *42:* 79–102

KATZ, B. 1948 The electrical properties of the muscle fiber membrane. Proc. Roy. Soc. B., *135:* 506–534.

TOBIAS, J. M. 1952 In: Trends in Physiology and Biochemistry, ed. E. S. G. Barron, p. 291. Academic Press, New York, N. Y.

USSING, H. H. 1949 Transport of ions across cellular membranes. Physiol. Rev., *29:* 127–155.

UTILIZATION OF ADDED SUGARS BY CHICK HEART FIBROBLASTS IN DIALYZED MEDIA[1]

MORGAN HARRIS AND PHYLLIS B. KUTSKY
Department of Zoology, University of California, Berkeley

FIVE FIGURES

INTRODUCTION

Nutritional studies on tissue cells *in vitro* have centered for many years on two main problems: (1) the nature of growth promoting agents in embryo extract (Baker and Carrel, '26; Fischer, '46; Davidson and Waymouth, '45), and (2) the development of synthetic media (Baker, '36; White, '46, '49; Morgan, Morton and Parker, '50). The effects of individual nutrients have received less attention and at present information is meagre or lacking on the specific roles of amino acids and proteins, carbohydrates, lipids, and vitamins for growth of cells in tissue culture. These questions have not been answered by the use of synthetic mixtures owing to the clear inadequacies at present of the best chemically defined media. As yet no known synthetic medium will promote protein synthesis *in vitro* (Gerarde, Jones and Winnick, '52) or even provide for maintenance of cell number (Evans, Shannon, Waltz, Sanford and Earle, '53).

The continuing lack of a suitable synthetic medium for nutritional studies has necessitated the employment of more indirect methods for securing information. One of these features the use of dialyzed media, an approach first introduced by Fischer in 1941 and recently developed to a further state of usefulness (Harris, '52a). This method involves the cultivation of explanted tissues in plasma, serum, and embryo extract, each simplified by prior dialysis against a balanced saline before combination for culturing. The characteristic deficiency syndrome observed with outgrowths in this basal medium represents a basis of assay for diffusible nutrients removed by the dialysis procedure. Dialyzed media have been used recently to investigate the role of bicarbonate for tissue cells *in vitro* (Harris, '51, '53b) as well as for a study of unknown nutrient factors occurring in alcoholic dialysates of chick embryo extract (Harris, '52b, '53a).

---

[1] Supported by grants from the Cancer Research Coordinating Committee, University of California.

In the present investigation similar techniques were employed to study the role of sugars in the nutrition of chick heart fibroblasts, using media dialyzed to remove free glucose. The results of these experiments provide information on the characteristics of outgrowth in the absence of free glucose, energy sources for cell proliferation, the availability of alternative carbohydrates, and the problem of adaptation to new substrates.

MATERIAL AND METHODS

A brief description will suffice for the preparation of media, dialysis procedures, and culture operations, since a detailed account of methods now in use has been published previously (Harris, '52a). Chicken plasma and chick embryo extract were prepared directly and horse serum obtained commercially. Each of these was dialyzed in 25 ml aliquots for 8 days against a sugar-free buffered saline (F-2 solution). The dialyzing fluid was changed daily and all procedures carried out under sterile conditions. As a check on the completeness of dialysis a series of sugar analyses was performed on stock dialyzed media and on incubated media blanks. For measurement the media were deproteinized with copper sulfate and sodium tungstate according to the method of Somogyi ('31), and the equivalent glucose concentration in the supernatant determined by titration with ceric sulfate as described by Giragossintz, Davidson and Kirk ('36). Reducing substances equivalent to 0.01 mg glucose/ml were found in supernatants from both incubated and unincubated media. These data are consistent with experimental results to be described below which indicate the essential absence of free glucose in unsupplemented stock media.

All cultures were carried out in Carrel D-3.5 flasks using fresh explants obtained from 12–15-day chick heart. In preparing the cultures 0.5 ml dialyzed plasma was added to each flask, mixed with 1.0 ml clotting fluid, and a single explant added just before coagulation took place. Subsequently a fluid phase was added consisting of 40% dialyzed horse serum, 15% dialyzed chick embryo extract (EE_{50}) and 45% glucose-free Gey's solution. Supplements to be tested were included in the saline fraction as were the following in amounts appropriate to give the corresponding concentrations in the completed medium: 100 units/ml sodium penicillin G (Merck), 50 units/ml dihydrostreptomycin (Pfizer) and 0.002% phenol red. The clotting fluid was similar to the supernatant except that serum was omitted and the concentration of other components increased 50% to adjust the composition of the solid phase to that of the supernatant. The cultures were incu-

TABLE 1

Growth indices for chick heart fibroblasts in dialyzed and undialyzed media

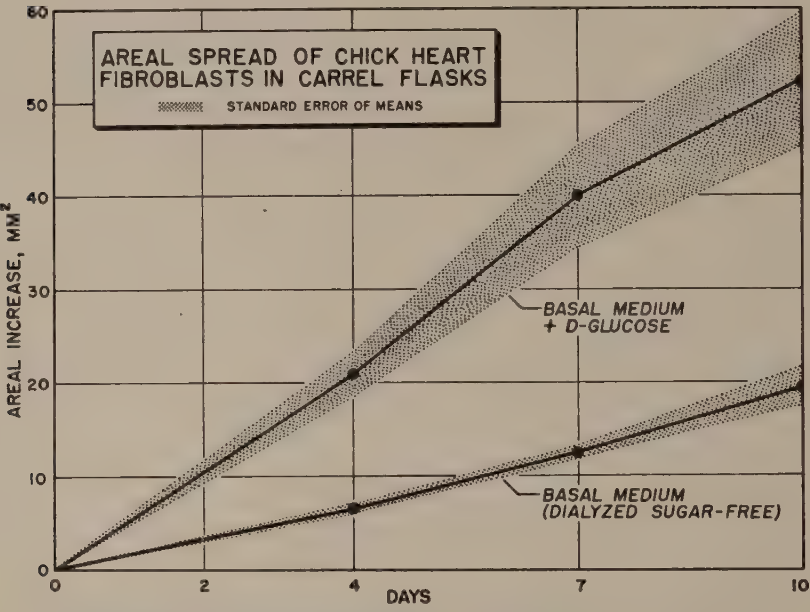
MEDIUM [1]	SUPPLEMENT	AREAL INCREASE PER EXPLANT AT 10 DAYS [2]	pH LEVEL AT 7 DAYS [3]	LACTIC ACID AT 7 DAYS [3]
		mm^2		*μg/ml*
Dialyzed glucose-free	....	19.6 ± 2.1	7.7	4.8
	0.15% D-glucose	52.4 ± 7.3	7.2	349
Undialyzed	....	128.7 ± 1.9	7.1	642

[1] *Total medium:* Chicken plasma clot + supernatant 40% horse serum, 15% chick embryo extract (EE_{50}), 45% Gey's solution (glucose-free where used in dialyzed media).

[2] Mean value and standard error (Snedecor, '46). Six cultures per series.

[3] pH and lactic acid determinations were made on pooled supernatants from pairs of cultures.

bated at 38°C. and supernatant fluids replaced at biweekly intervals without washing the cultures. Routine areal tracings were made with a projectoscope and after measurement with a planimeter the data were treated statistically.



* SUPERNATANTS: 40 % HORSE SERUM, 15 % CHICK EMBRYO EXTRACT (1:1), 45 % GEY'S SOLUTION (GLUCOSE-FREE).
* CONCENTRATION OF ADDED D-GLUCOSE: 1.5 MG./ML. MEDIUM.

Figure 1

Outgrowths in sugar-free media and in the presence of added glucose

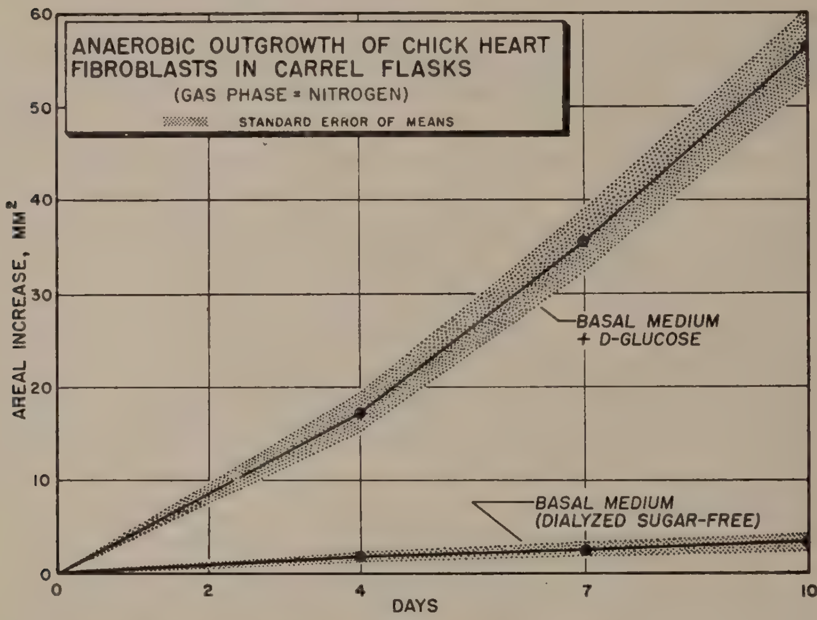
An initial group of experiments was performed to study the outgrowth of chick heart fibroblasts in media dialyzed to remove free sugar. These cultures were compared with others established in the same media but supplemented with 0.15% D-glucose. A number of clear-cut differences were immediately apparent between the two types of cultures. The absence of free glucose did not prevent cell proliferation and migration, and a compact, sustained outgrowth occurred (fig.

4). The areal increase, however, was markedly less than that obtained when the medium was supplemented with D-glucose (figs. 1, 4, 5) and an even greater differential was found when comparison was made with undialyzed media (table 1). Explants in sugar-free media continued to proliferate vigorously after three subcultures at intervals of two weeks.

The appearance of cells in the outgrowth zone was equally characteristic for cultures maintained without added glucose. After a few days the marginal cells became conspicuously branched and giant cells appeared sporadically in this region. Throughout the outgrowth zone the cells exhibited a pale, granular appearance but were without the prominent accumulations of fat vacuoles and refractile surfaces usually seen in cells cultivated in dialyzed media (Harris, '52a) and which were characteristic here of outgrowths in sugar-free media supplemented with D-glucose.

The most conspicuous difference between the series in question was evident from the presence of phenol red as an indicator in the assay media. While cultures supplemented with D-glucose showed the usual fall in pH levels over a 10-day culture period, those maintained in sugar-free media remained at the same pH level through this interval. These observations were routinely verified by measurements with a Beckman pH meter, and led to the determination of lactic acid in the supernatants from 7-day cultures. The media were deproteinized with copper sulfate and calcium hydroxide, and lactic acid determined in the supernatants by the method of Barker and Summerson ('41) using a Beckman Model DU spectrophotometer. From the results shown in table 1 it is apparent that glycolysis is essentially absent in sugar-free media although extensive production of lactic acid occurs if D-glucose is added. In undialyzed media the level of glycolysis is still greater.

As a control measure, pH and lactic acid levels were also determined in a series of blank cultures, set up without explants and incubated for 7 days. Media dialyzed glucose-free were employed and in some cases were used without supplementation while in others 0.15% D-glucose or glucose + 0.001% adenosine triphosphate were added. In all of these series the pH level remained constant over the 7-day incubation period and the lactic acid values in the terminal supernatants did not differ essentially. It is thus apparent that glycolysis in dialyzed media occurs only in the presence of explants and available sugar, and that a series of specific criteria including sugar depletion, pH drop, and lactic acid production as well as cell morphology and increase in area may be used as indices for measuring the effect of carbohydrate substrates when added to sugar-free assay media.



* CONCENTRATION OF ADDED D-GLUCOSE: 1.5 MG./ML. MEDIUM

Figure 2

Energy sources for outgrowth

The results outlined in the preceding paragraphs indicate that proliferation and migration of chick heart fibroblasts

Effect of added sugars on cultures of chick heart fibroblasts in sugar-free dialyzed media[1]

SUPPLEMENT[2]	CONCENTRATION IN MEDIUM	DECREASE IN SUGAR CONCENTRATION AT 7 DAYS[3]	pH LEVEL IN SUPERNATANT AT 7 DAYS	LACTIC ACID AT 7 DAYS	AREAL INCREASE PER EXPLANT AT 10 DAYS OVER BASAL OUTGROWTH[3]
	mg/ml	*mg/ml*		*μg/ml*	*mm²*
Unsupplemented basal medium	...	..	7.7	4.8	..
D-glucose	1.50	0.34 ± 0.02	7.2	349	32.9 ± 7.5
L-glucose	1.50	0.03 (pooled sample)	7.7	3.3	2.5 ± 7.3
D-fructose	1.50	0.15 ± 0.01	7.5	67	25.2 ± 9.2
D-mannose	1.50	0.45 ± 0.02	7.2	317	39.2 ± 5.2
D-galactose	1.50	0.02 ± 0.01	7.7	7.4	— 11.2 ± 2.3
D-ribose	1.25	0.02 ± 0.00	7.6	3.8	1.6 ± 5.9
D-arabinose	1.25	0.00 (pooled sample)	7.7	11.1	— 4.4 ± 3.9
D-xylose	1.25	0.02 ± 0.01	7.7	6.0	— 3.1 ± 2.4
D-erythrose	1.00	..	7.4	4.0	— 8.8 ± 5.3
D-sucrose	2.85	0.02 ± 0.02	7.7	15.7	3.2 ± 2.9
D-lactose	2.85	0.02 ± 0.03	7.6	4.3	4.1 ± 3.3
D-maltose	2.85	..	7.1	360.0	25.2 ± 3.1
D-raffinose	4.95	0.00 (pooled sample)	7.7	7.5	— 8.7 ± 5.5
Glycogen	5.00	..	7.0	370	54.5 ± 6.5

[1] Total medium: dialyzed chicken plasma clot + supernatant 40% dialyzed horse serum, 15% dialyzed chick embryo extract (EE_{50}), 45% glucose-free Gey's solution.

[2] Six cultures per series. Glucose determinations and areal measurements on individual cultures except as noted, pH and lactic acid determinations on pooled supernatants from pairs of cultures.

[3] Mean value and standard error (Snedecor, '46).

can occur in the absence of glycolysis as an energy source and suggest that oxidative utilization of non-carbohydrate materials supplies the energy requirement in this case. To test this suggestion cultures were established in sugar-free media and in glucose-supplemented media but with a gas phase of nitrogen instead of air. Comparable pH levels were maintained for both series. The striking differences obtained are shown in figure 2. In sugar-free media outgrowth was suppressed under these anaerobic conditions. A few cells migrated out from the explants but a growth zone as such was absent. This minimal response may represent a small residual oxygen tension in the nitrogen used. On the other hand the addition of D-glucose permitted a flourishing outgrowth to develop under anaerobic conditions. These results suggest that while both glycolysis and aerobic oxidation may provide energy for proliferation and migration of chick heart fibroblasts under normal conditions, either mechanism may be adequate as an energy source.

Alternative carbohydrate substrates

The clear-cut results of adding D-glucose to cultures growing in sugar-free media provided a yardstick for measuring the utilization of other carbohydrates by chick heart fibroblasts. A series of monosaccharides was therefore tested by supplementation at molar strengths equivalent to 0.15% D-glucose. This list included L-glucose, D-fructose, D-mannose, and D-galactose as well as D-ribose, D-xylose, D-arabinose, and D-erythrose. Cultures containing each of the added sugars were observed microscopically over a 10-day period, routine pH data and areal tracings obtained, and analyses for sugar and lactic acid carried out on the supernatants. In these experiments sharp differences were observed in the availability of the various sugars for use by chick heart fibroblasts. Of the monosaccharides tested only D-mannose proved capable of duplicating the effects of D-glucose at equivalent molar concentrations (table 2). Cultures supplemented with mannose closely resembled those supplemented with D-glucose with regard to the appearance of cells in the outgrowth zone and also in pH decline, sugar decrease, and formation of lactic acid. Added D-fructose was also utilized but less efficiently than mannose (table 2). D-galactose was not utilized and in addition proved conspicuously inhibiting at 0.15% concentrations. Outgrowths in the presence of galactose were much more sparse than in the sugar-free basal medium and after a few days took on a bizarre, heterotypic appearance. Large, blunt, granular projections were prominent in parts of the outgrowth zone while in other areas the cells developed

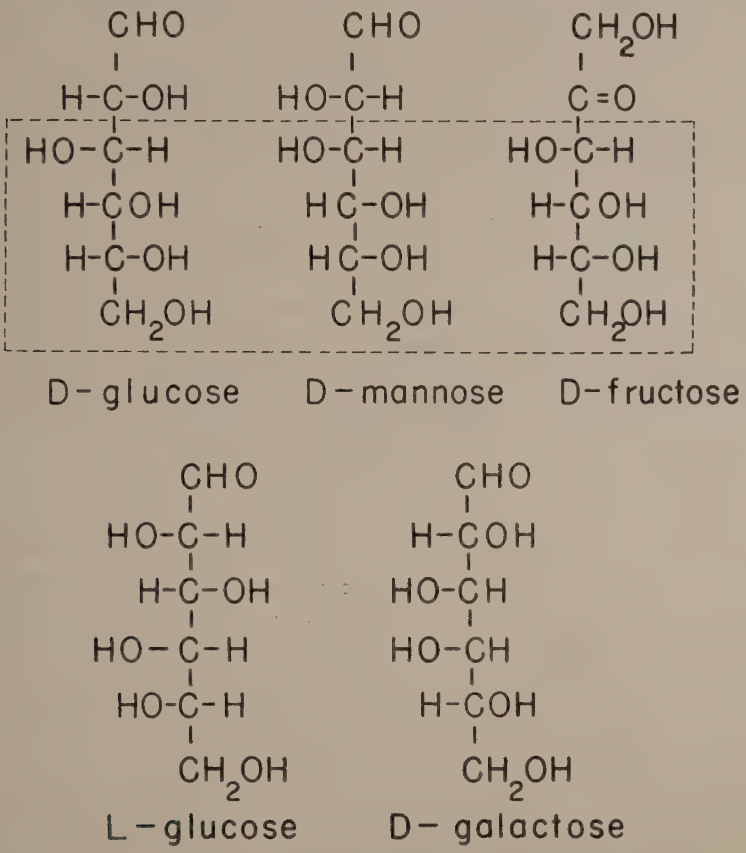


Fig. 3 Comparative structure of monosaccharides.

a foamy appearance or became highly arborized. Despite these signs of inhibition the galactose cultures slowly increased in size over a period of weeks, could be subcultured repeatedly and were maintained for many months (see next section). L-glucose, D-ribose, D-xylose, D-arabinose, and D-erythrose were not inhibiting but proved completely inert by microscopic examination and other criteria used. Cultures supplemented with these sugars closely resembled those maintained in sugar-free media. The high selectivity of the cells for D-glucose, D-mannose, and D-fructose can be explained on a stereochemical basis (fig. 3). Each of these displays a pattern of comparable asymmetry on carbon atoms 3–5, and this feature is lacking in the hexoses L-glucose and D-galactose found to be inert in the present experiments. It is interesting, however, that if the stereochemical configuration represented by D-glucose is shortened by the removal of the first carbon atom (D-arabinose) or by removal of both first and second carbons (D-erythrose) the resulting sugars are inert. There was no indication that free pentoses or tetroses can be utilized as such by chick heart fibroblasts.

In addition to the monosaccharides described above, cultures were also supplemented with D-sucrose, D-lactose, D-maltose, D-raffinose, and glycogen (table 2). All were completely inert except maltose and glycogen which gave strongly positive tests. To study the mechanism of utilization of these materials, the fluid phases from cultures supplemented with maltose and glycogen respectively were treated with phenylhydrazine to form osazones from the sugars present. Large amounts of glucosazone crystals were observed in both cases. This finding led to the incubation of blank media containing glycogen or maltose followed by formation of osazones. The results showed clearly that maltose and glycogen are hydrolyzed to glucose in the absence of explants, and that the amylase apparently responsible occurs not only in chick embryo extract but also in chicken plasma and horse serum as well. It is apparent, therefore, that the question of direct utilization of maltose and glycogen as such by explants cannot be answered by the assay technique appropriate for the monosaccharides.

Long term culture in the presence of inert substrates

The foregoing experiments show that for short periods of cultivation the utilization of different sugars by chick heart fibroblasts is sharply restricted. It seemed possible, however, that adaptive changes might be observed with cultures maintained for longer periods in the presence of an inert substrate. Accordingly a strain of chick heart fibroblasts was established in sugar-free dialyzed media supplemented with 0.125% D-arabinose, and was maintained for 6 months with biweekly change of the fluid phase. These cultures grew slowly but steadily so that subculture was required every 4 or 5 weeks. The strain was continued at each subculture by inoculating 6 new flasks with explants obtained from a single outgrowth of the preceding series. The central fragment of the old outgrowth was discarded.

Outgrowths in arabinose treated cultures early assumed a remarkably uniform and characteristic appearance. The cells, especially at the periphery, became enlarged and conspicuously arborized, with an intricate network of fine branching. This curious growth pattern was quite different from the usual appearance of fibroblasts in undialyzed media or in dialyzed media containing glucose. It is worth mentioning, however, that the arborized cells were strikingly similar to the bizarre stellate cells observed by Parker ('33) in fibroblast cultures maintained for long periods in dilute serum, and which are shown by high power photomicrographs in Plate 10 of his paper. The appearance of the photographed cells is identical to that of typical elements from the arabinose cultures of the present experiments. This resemblance is particularly interesting since Parker did not use dialyzed materials and the composition of his medium was quite different.

The growth pattern just described was maintained in the arabinose cultures without essential change over long periods.

Grossly the outgrowths remained clear and pale, nearly transparent, without conspicuous inclusions except irregular granulation. They remained dense and healthy in appearance, without signs of necrosis or degeneration throughout the 6 months period. The pH level remained high in these cultures and there was no sign of increased acid production. At the end of 6 months experiments were set up to compare the arabinose-treated fibroblasts with a strain of chick heart fibroblasts carried in undialyzed media for 105 days with routine subculture. Explants obtained from outgrowths of these two strains were cultivated separately in undialyzed media, in dialyzed sugar-free media without supplementation, in dialyzed media containing D-glucose, and in dialyzed media + D-arabinose. The results of these experiments are shown in table 3. When compared to the strain cultivated in normal

TABLE 3

Comparison of outgrowth in cultures of normal[1] *and arabinose-treated fibroblasts*[2]

CULTURE MEDIUM[3]	SOURCE OF FIBROBLASTS	pH LEVEL IN SUPERNATANTS AT 10 DAYS	AREAL INCREASE PER EXPLANT AT 10 DAYS[4]
			mm^2
Undialyzed	Normal strain	7.08	98.1 ± 4.6
	Arabinose-treated	7.09	89.3 ± 4.2
Dialyzed sugar-free no supplement	Normal strain	7.62	39.4 ± 2.9
	Arabinose-treated	7.63	25.2 ± 1.1
Dialyzed + 0.15% D-glucose	Normal strain	7.28	55.8 ± 2.2
	Arabinose-treated	7.33	33.6 ± 4.2
Dialyzed + 0.125% D-arabinose	Normal strain	7.59	35.0 ± 2.7
	Arabinose-treated	7.63	21.4 ± 2.2

[1] Normal fibroblasts: from fifth passage of chick heart fibroblasts maintained 105 days in undialyzed media.

[2] Arabinose-treated fibroblasts: from fifth passage of chick heart fibroblasts maintained 6 months in sugar-free dialyzed media supplemented with 0.125% D-arabinose.

[3] Total medium: Solid phase of dialyzed chicken plasma + supernatant of 40% dialyzed horse serum, 15% dialyzed embryo extract (EE_{50}) + 45% Gey's solution.

[4] Mean value and standard error (Snedecor, '46). Six cultures per series.

media the arabinose strain of fibroblasts tested in the presence of arabinose showed neither a heightened production of acid nor increased surface area. On the other hand the arabinose strain retained after 6 months the capacity to respond to D-glucose and to the dialysate factors present in undialyzed media. In these media the large arborized cells were rapidly replaced by a population of smaller fibroblasts with the usual blunt or spindle-shaped appearance. Except in undialyzed media the arabinose-treated fibroblasts showed a lesser proliferation than the untreated normal strain, but this probably reflects merely a lower cell density in the test explants.

In another series of experiments a strain of chick heart fibroblasts was maintained 6 months in sugar-free media supplemented with 0.15% D-galactose. There was no sign of increased acid production and the galactose concentration remained unchanged in the presence of outgrowth. These cultures, however, exhibited an extremely heteromorphic cell picture and the outgrowths did not stabilize into a uniform growth form. The initial outgrowths showed severe inhibition with cells predominantly massed into blunt, granular, vacuolated projections. Later a few pale cells could be occasionally observed and in subsequent subcultures these became more prominent and formed arborized networks comparable to those of the arabinose cultures but much less regular and extensive. Some of these networks persisted indefinitely; others became granular and degenerated. In some cultures conspicuous strands of minute rectangular elements extended out from the explants or were observed in small groups near the outgrowths in the surrounding medium. These cells, possibly macrophages, were obtained in pure form in subculture but in numbers insufficient to establish a strain for experiment. In general, the phenomena observed in the galactose cultures suggested morphologic adaptation to the inhibiting effects of galactose, although the adjustments observed were partial at most.

It may also be mentioned that other strains of fibroblasts were cultured for 6 months in sugar-free media without supplementation and in media containing 0.285% sucrose. In general these resembled the arabinose-treated cultures although characteristic small differences were found. Thus the sucrose cultures grew more rapidly and showed an increased tendency to liquefy the plasma clot; the cell picture in the outgrowth zone was more varied although largely made up of arborized cells. Outgrowths in sugar-free media alone were more sparse than with added arabinose; the cell population was somewhat more open and less uniform than the massed networks of arborized cells seen with arabinose.

DISCUSSION

The experiments of Astrup, Fischer and Oehlenschlager ('47), reported briefly in the course of another investigation, appear to represent the only other study on the utilization of specific sugars by cells *in vitro*. These workers employed Ringer-dialyzed media supplemented with malt extract which had been fermented with yeast to remove sugar. The cells were unable to survive in this glucose-free medium although proliferation occurred in the presence of added glucose. It may be pointed out, however, that failure of outgrowth in Ringer-dialyzed media has recently been correlated with reduced pH and bicarbonate levels rather than nutritional deficiencies as such (Harris, '52a), and that protein denaturation occurring as a result of pH drop renders Ringer-dialyzed media inferior for assay even if pH and buffer levels are subsequently raised. It appears that these or other inadequacies of Ringer-dialyzed media, rather than the absence of glucose, are responsible for the failure of the cells to survive as noted. In the present experiments, using media prepared by dialysis against a buffered saline, we have regularly obtained stable basal outgrowths in glucose-free media, and these cultures continued to survive and proliferate for many months. That this outgrowth was not caused by residual sugar is indicated by direct analysis of the culture media for glucose content, by the absence of lactic acid production during the culture period, and by the distinctive morphological differences of cell strains cultivated in the presence and absence of glucose or other utilizable sugar.

Other data reported by Astrup et al. in the paper mentioned above are consistent with present findings. Glucose, mannose, and fructose supported proliferation while outgrowth failed if pentoses, sucrose, or lactose were added instead. On the other hand glycogen was inert and maltose showed only a limited activity, whereas in the present experiments both were equivalent in effect to glucose. It seems possible that this difference may reflect an inactivation during Ringer-dialysis of the amylase found responsible in the present investigation for the extracellular hydrolysis of glycogen and maltose to glucose. Galactose was reported to show some activity but this view was based solely on areal spread; no analyses of culture media were made to determine sugar content or lactic acid. We have consistently found galactose not only inert by all of these criteria but actively inhibiting as well.

The role of specific sugars in embryonic development has been extensively studied by Spratt ('48, '49, '50, '52). Chick blastoderms were explanted at an early stage to a saline-agar substrate and the nutrient requirements for development determined by appropriate supplementation of this culture medium. His results afford a number of interesting examples of dissociation between the processes of morphogenesis, differentiation and growth, and indicate that the nutrient requirements may differ for each process as well as for specific organs and tissues. It is clear, for example, that an exogenous source of glucose or other utilizable carbohydrate must be provided for morphogenesis and differentiation to occur in explanted blastoderms (Spratt, '48). In sugar-free media these processes cease abruptly and whatever structural organization attained is rapidly lost with a diffuse dispersal of cells. The dispersed cells, however, retain the ability for proliferation as far as this point was studied, a finding which is consistent with growth of chick heart fibroblasts in sugar-free media in the present experiments.

By substituting other carbohydrates for glucose in the culture medium Spratt ('49) found that the majority of these including pentose sugars, sucrose, and lactose were inert. Fructose and mannose supported morphogenesis and differentiation, also galactose if used in high concentration (200–800 mg %). This observation is interesting since our own findings indicate that galactose at 150 mg % does not stimulate growth of chick heart fibroblasts. It is possible that other tissues in the chick embryo can utilize galactose or convert it to glucose (cf. adult liver), or that the effect of galactose on morphogenesis does not parallel its effects on growth.

The possibility of adaptive change in the carbohydrate requirements of cell populations *in vitro* remains as an interesting problem. As a whole the long-term experiments described in the present paper have failed to reveal clear-cut adaptive shifts in the presence of inert sugars although alterations in cell morphology were noted. It would be desirable, however, to extend this exploration under cultural conditions more strongly favoring adaptive change. For example, in the present experiments the cell populations were maintained aerobically and thus could presumably satisfy energy requirements by oxidative breakdown of non-carbohydrate sources. There was no strong selection pressure favoring a variant which might utilize the inert substrate. It would be interesting to perform similar experiments under anaerobic conditions where the presence of utilizable sugar is an essential requirement for cell proliferation.

ACKNOWLEDGMENTS

It is a pleasure to give recognition to the cooperation and technical assistance of Mr. Ronald Branson, Mr. John Fordon, Mr. Robert Janson, and Miss Mirian Lieberman in carrying out this investigation.

SUMMARY

1. Plasma, serum, and embryo extract were dialyzed glucose-free and used as a basal medium for determining the

effects of added sugars on the outgrowth of chick heart fibroblasts. Areal spread, cell morphology, production of lactic acid, and depletion of sugar concentration were employed as criteria for utilization.

2. Cultures maintained aerobically in glucose-free media do not produce lactic acid and show a minimal but stable outgrowth, with a characteristic pattern of cell structure. The addition of D-glucose restores glycolysis, increases surface area, and alters the appearance of cells in the outgrowth zone.

3. Under anaerobic conditions outgrowth is suppressed in glucose-free media but takes place vigorously in the presence of added D-glucose. The energy requirement for the proliferation of chick heart fibroblasts may be obtained either by glycolysis or aerobic oxidation.

4. D-mannose and D-fructose are utilized by chick heart fibroblasts but L-glucose, D-galactose, D-sucrose, D-lactose, and three pentose sugars are inert. Glycogen and D-maltose are hydrolyzed to glucose by extracellular enzymes occurring in the assay medium.

5. Long-term cultivation of chick heart fibroblasts in the presence of D-arabinose, D-galactose, and D-sucrose, respectively, revealed no clear-cut evidence of adaptive changes although alterations in cell morphology were noted.

LITERATURE CITED

ASTRUP, T., A. FISCHER, AND V. OEHLENSCHLAGER 1947 Protein metabolism of tissue cells in vitro. 5. The accessory growth substances in sugar-free malt extracts. Acta. Physiol. Scand., *13:* 267-276.

BAKER, L. E. 1936 Artificial media for the cultivation of fibroblasts, epithelial cells, and monocytes. Science, *83:* 605–606.

BAKER, L. E., AND A. CARREL 1926 Action on fibroblasts of the protein fraction of embryonic tissue extract. J. Exp. Med., *44:* 387–395.

BARKER, S. B., AND W. H. SUMMERSON 1941 The colorimetric determination of lactic acid in biological material. J. Biol. Chem., *138:* 535–554.

DAVIDSON, J. N., AND C. WAYMOUTH 1945 The nucleoprotein content of fibroblasts growing in vitro. Biochem. J., *39:* 188–199.

EVANS, V. J., J. E. SHANNON, JR., H. K. WALTZ, J. C. BRYANT, K. K. SANFORD and W. R. EARLE 1953 A quantitative evaluation of chemically defined media for culturing of fibroblasts in vitro. J. Nat. Cancer Inst., *13:* 773.

FISCHER, A. 1941 Die Bedeutung der Aminosaüren für die Gewebezellen in vitro. Acta Physiol. Scand., *2:* 143–188.

——— 1946 Biology of tissue cells. Hafner Publishing Co., New York. 348 pp.

GERARDE, H. W., M. JONES AND T. WINNICK 1952 Protein synthesis and amino acid turnover in tissue culture. J. Biol. Chem., *196:* 51–58.

GIRAGOSSINTZ, G., C. DAVIDSON AND P. L. KIRK 1936 The micro determination of blood sugar by ceric sulfate titration. Mikrochemie, *21:* 21–34.

HARRIS, M. 1951 Bicarbonate as an essential growth factor for chick heart fibroblasts in dialyzed media. Anat. Rec., *109:* 372.

——— 1952a The use of dialyzed media for studies in cell nutrition. J. Cell. and Comp. Physiol., *40:* 279–302.

——— 1952b Growth factors in alcoholic extracts of chick embryos. Growth, *16:* 215–230.

——— 1953a Partial purification of growth factors in the dialyzable fraction of chick embryo extract. Growth, *17:* 147–162.

——— 1953b The role of bicarbonate for outgrowth of chick heart fibroblasts in vitro. J. Exp. Zool. (in press).

MORGAN, J. F., H. J. MORTON AND R. C. PARKER 1950 Nutrition of animal cells in tissue culture. I. Initial studies in a synthetic medium. Proc. Soc. Exp. Biol. Med., *73:* 1–8.

PARKER, R. C. 1933 The races that constitute the group of common fibroblasts. II. The effect of blood serum. J. Exp. Med., *58:* 97–113.

SNEDECOR, G. W. 1946 Statistical methods applied to experiments in agriculture and biology. The Collegiate Press, Ames. 485 pp.

SOMOGYI, M. 1931 The use of copper and iron salts for the deproteinization of blood. J. Biol. Chem., *90:* 25–30.

SPRATT, N. 1948 Development of the early chick blastoderm on synthetic media. J. Exp. Zool., *107:* 39–64.

——— 1949 Nutritional requirements of the early chick embryo. I. The utilization of carbohydrate substrates. J. Exp. Zool., *110:* 273–298.

——— 1950 Nutritional requirements of the early chick embryo. II. Differential nutrient requirements for morphogenesis and differentiation of the heart and brain. J. Exp. Zool., *114:* 375–402.

——— 1952 Metabolism of the early embryo. Ann. N. Y. Acad. Sci., *55:* 40–49.

WHITE, P. R. 1946 Cultivation of animal tissues in vitro in nutrients of precisely known composition. Growth, *10:* 231–289.

——— 1949 Prolonged survival of excised animal tissues in vitro in nutrients of known composition. J. Cell. and Comp. Physiol., *34:* 221–242.

PLATE

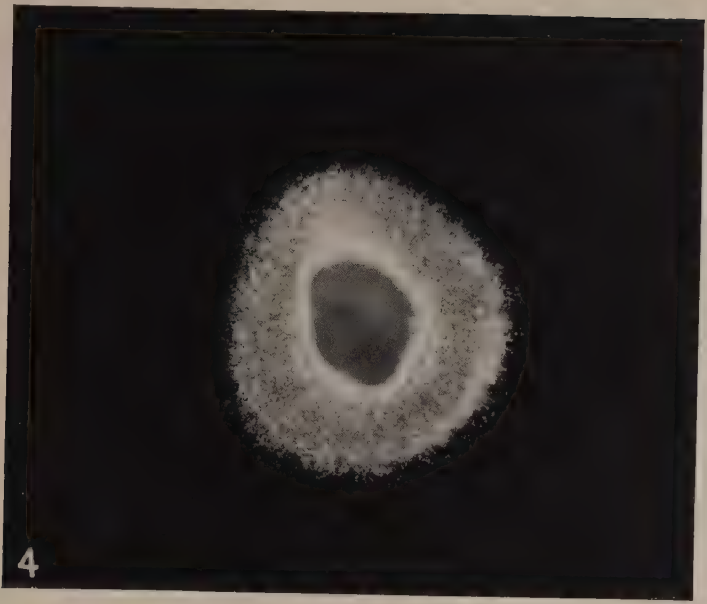
PLATE 1

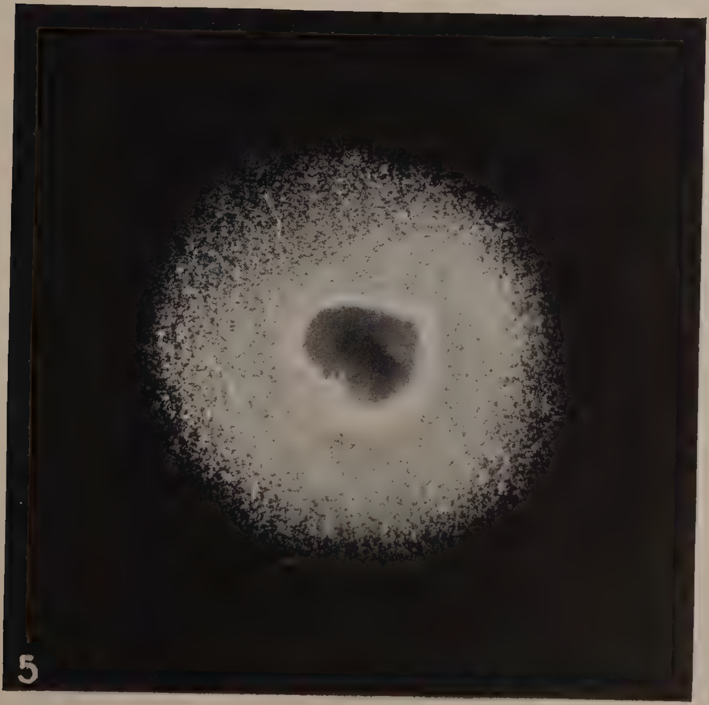
EXPLANATION OF FIGURES

Photomicrographs of 7-day cultures of chick heart fibroblasts in Carrel flasks.

4 Outgrowth in media dialyzed glucose-free and without added sugar. × 7.5.

5 Outgrowth in same medium supplemented with 0.15% D-glucose. × 7.5.
Photomicrographs taken by Mr. Victor Duran.


4


5

STUDIES ON CA^{45} TRANSPORT IN CRAYFISH NERVE

SIDNEY SOLOWAY, J. H. WELSH AND A. K. SOLOMON
Biophysical Laboratory, Harvard Medical School and Biological Laboratories, Harvard University

TWO FIGURES

The present studies have been undertaken as a preliminary investigation of the transport of Ca^{45} in crayfish chela nerves. Our results show that the loss of Ca^{45} can be described by a curve consisting of two exponential terms, and we have investigated the effects of changes of Ca ion concentration and temperature on the constants in these terms.

METHOD

The incubating medium for the crayfish nerve was van Harreveld's ('36) solution in which the normal calcium had been replaced by Ca^{45}. This solution was chosen as bathing medium in these experiments because van Harreveld had found it to be the optimal bathing solution for crayfish nerves, in which they maintained their normal excitable condition for periods as long as 25 to 35 hours. Its composition is given in table 1. The nerve used throughout this study consisted of the chela axons of the crayfish, *Cambarus virilis*. These axons are thinly myelinated, and essentially free of connective tissue. The nerve bundle, which varied from 0.5 to 1.0 cm in length, was composed of a few large axons and a number of small sensory fibers. All experiments were conducted in a constant temperature bath held at 15°C. ± 1°C. with the exception of those to determine the temperature coefficient.

The nerves were incubated initially in a 6 ml volume of Ca^{45} van Harreveld's solution for exactly one hour. They were then washed for 10 minutes in a large excess of non-radioactive van Harreveld's solution. The nerve was then transferred to an aluminum planchet containing 1 ml of the solution under test. It was allowed to remain in this planchet for a length of time sufficient to yield to the surrounding medium an amount of Ca^{45} which could be easily measured. The nerve was then transferred to a second planchet, and the process repeated. This procedure continued until a total of about 8 planchets had been employed. The nerve was then coiled at the bottom of a 9th planchet and dried under a heat lamp, along with the entire set of planchets. The radioactivity of each planchet was then measured. The relative Ca^{45} content of the nerve at times throughout the washing periods could be determined from the knowledge of the relative Ca^{45} content of the nerve at the end of the experiment and the Ca^{45} lost to each planchet during the washing periods.

TABLE 1

Composition of van Harreveld's solution

	gm/liter	*mmol/liter*
NaCl	12.0	206.0
$CaCl_2$ [1]	1.5	13.5
KCl	0.4	5.4
$MgCl_2 \cdot 6H_2O$	0.533	2.6
$NaHCO_3$	0.2	2.4
pH	7.5	

[1] In Ca^{45} van Harreveld's solution all the $CaCl_2$ was replaced by high specific activity $Ca^{45}Cl_2$.

The one hour incubation period was chosen because preliminary experiments indicated, within the rather wide variability of the results, that the radioactivity in the nerve had reached an approximate maximum at this time. It is interesting to note in this connection that Rothenberg ('50) has found that the Ca^{45} activity in squid axoplasm reaches a maximum in 50 minutes. At 100 minutes Rothenberg's data show less Ca^{45} in the axoplasm than at 50 minutes, suggesting that the axon may be losing Ca. In our experiments, on the other hand, the single observation (10.9 counts/min. mg wet nerve) at 90 minutes' incubation is almost as high as the highest value observed at 50 minutes (11.6 counts/min. mg wet nerve) and the difference between them is far less than the normal variability in the uptake data.

Since no direct measurements of the total nerve Ca were made, we can only infer that the nerve was in a steady state with respect to its Ca content. By making use of van Harreveld's observation that nerves in his medium retain their normal excitable condition for periods as long as 25 hours, we can set an approximate upper limit on the rate of loss of Ca from the nerve. If the nerve were losing Ca at a uniform rate such that half its Ca were lost in 6 hours, there would remain only 6.25% of the initial Ca at the end of a 24 hour period. Such a low Ca content would hardly seem consonant with a normally excitable nerve such as van Harreveld observed. Consequently we would expect the leakage to be slower than that, or in other terms to be less than 12% of the Ca content per hour. Since in our experiments, as will be seen, half the Ca^{45} was exchanged in about 30 minutes, it seems unlikely that this process represents Ca leakage exclusively. Furthermore the half-life for the slower process which we have observed in crab nerve is two and one-half hours, which is fast compared to the minimum half-life of 6 hours for the leakage as estimated above. Consequently, our results will be interpreted on the basis of an approximate steady state for the Ca content in the nerve, but with the explicit reservation that confirmation of this point awaits further study.

Measurement of radioactivity

Ca^{45} which has a 152 day half-life emits a 0.255 Mev beta ray (National Bureau of Standards Circular 499). It has been supplied by the Atomic Energy Commission from Oak Ridge, as carrier free Ca^{45}Cl$_2$ in acid solution. The radioactivity was measured with an end-window Geiger counter, and the radiochemical purity was checked by absorption measurements. In view of the long half-life, no corrections are necessary for disintegration. Also, in view of the small amount of solid material in van Harreveld's solution, no correction has been required for self absorption in any of the planchets, except for the one containing the coiled nerve. Here, though a correction would have been desirable, it was not possible to make one, since no practical means were available for measuring the area occupied by the nerve which was of the order of 0.1 cm². This may have contributed in a large measure to the variability of our results. A variety of unsuccessful attempts were made to prepare uniform planchets containing the Ca^{45} of the nerve. These included acid and alkali digestion of the nerve, on glass or gold plated planchets. All of these treatments suffer from certain inherent disadtages such as a small flow of the glass planchet when it was heated to promote digestion of the nerve, or the breakdown of the gold plate when concentrated HNO_3 formed aqua regia with chloride ions from the nerve bundle.

TABLE 2

Record of experiment with nerve 25

Into Ca^{45} van Harreveld solution 3: 35
removed 4: 35
Rinsed in normal van Harreveld's twice from 4: 35 to 4: 45
Into first planchet 4: 45

PLANCHET NUMBER	TIME INTERVAL IN PLANCHET	ELAPSED TIME SINCE REMOVAL FROM Ca^{45}	Ca^{45} ACTIVITY IN PLANCHET	TOTAL ACTIVITY REMAINING IN NERVE
	min.	*min.*	*cts/min.*	*cts/min.*
1	5	10	785	4068
2	5	15	512	3279
3	5	20	361	2767
4	5	25	289	2406
5	20	30	602	2117
6	30	50	267	1515
7	30	80	116	1248
8	30	110	85	1132
9	plated	140	1047	1047

Nerve responds to electrical stimulation.

EXPERIMENTAL RESULTS

The normal control at 15°C. The nerve at all times during this experiment was exposed to the ionic environment of van Harreveld's solution, not only during its Ca^{45} incubation, but also during its initial washing and during the aluminum

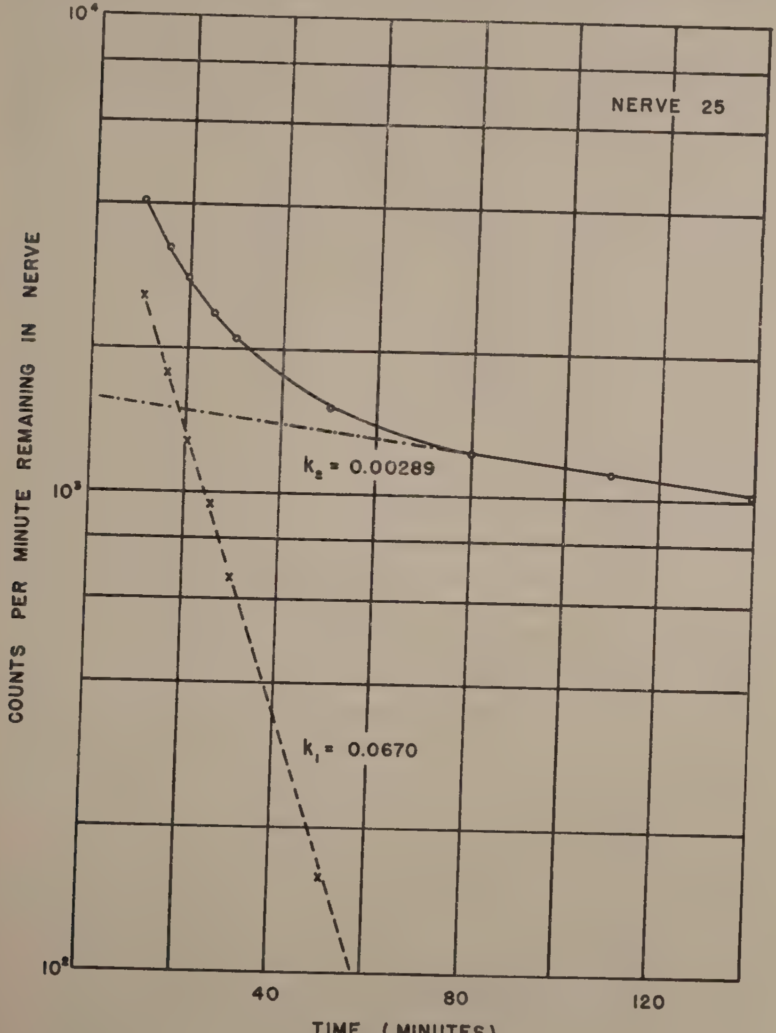


Figure 1

planchet series. Table 2 is a record of a typical experiment. The final column gives the integrated Ca^{45} content of the nerve determined from the sum of the Ca^{45} of the coiled nerve and the amount lost to the succeeding planchets. Figure 1 shows the activity of the nerve in this experiment plotted logarithmically against time. It can be seen that the loss of activity from the nerve can be described by an equation of the form:

TABLE 3

Normal outward flux of Ca^{45}

NERVE	$k_1 \times 10^2$/min.	$k_2 \times 10^3$/min.	a/b
14	8.55	5.26	3.5
15	8.71	2.86	2.5
16	9.34	3.33	4.5
17	11.20	8.47	4.5
18	10.60	5.16	9.4
25	6.70	2.89	3.2
26	12.50	1.36	1.3
42	8.85	2.35	6.3
170	5.05	8.95	3.0
171	8.41	5.82	3.4
180	7.20	7.14	2.7
Average	8.83	4.87	4.0
Standard deviation	2.1	1.4	2.2

$$R = ae^{-k_1t} + be^{-k_2t} \qquad (1)$$

where R is the amount of Ca^{45} in the nerve at time t following removal of the nerve from the incubating medium. In other experiments the process was followed for 200 minutes with no indication that further terms were needed in equation (1) to describe the data. However, in one experiment (nerve 140) a single point at 200 minutes would suggest that the loss of Ca^{45} at that time is less than would be predicted from equation (1). Consequently equation (1) should be considered to be valid only for periods less than 200 minutes.

Table 3 gives the values for the two constants k_1 and k_2 and the ratio a/b for 11 normal nerves. The average values of

$8.83 \pm 2.1 \times 10^{-2}$/min. obtained for k_1 and $4.87 \pm 1.4 \times 10^{-3}$/min. obtained for k_2 correspond to half-lives of 7.9 and 152 minutes respectively, for the two components of the curve.

Having established values of the constants k_1 and k_2, it was of interest to see whether the exchange was associated with vital processes of the nerve. The nerves were therefore denatured in two ways and their calcium exchange studied.

Two nerves were separately heated in Ca^{45} van Harreveld's solution at 100°C. for 30 minutes. They were then washed out with non-radioactive van Harreveld's and treated from this point on in the normal fashion. Although it was found that the loss of Ca^{45} from these denatured nerves again followed two straight lines, the slope of k_2, as given in table 4 has increased by a factor of 5 above normal while k_1 is in the normal range. In this case, high temperature denaturation of the nerve has considerably altered one of the indices of normal calcium exchange.

TABLE 4

Effects of denaturation and tying nerve ends on Ca^{45} outward flux

NERVE	CONDITIONS	$k_1 \times 10^2$/min.	$k_2 \times 10^3$/min.	a/b
172	Steam denatured	11.5	20.4	6.8
173		8.2	23.0	5.9
129	Distilled water	0.59	...	..
130		0.75	...	..
3-T	Tied ends	9.03	3.2	3.0

As a second test, nerves were incubated in the Ca^{45} solution normally, and the initial 10-minute washing, as well as the washing in the subsequent planchet series, was carried out in distilled water. The initial 10-minute washing changed the physical character of the nerve, giving it the consistency of a stiff white thread. Table 4 shows that the two slopes evident in the normal nerve have now given way to a single one. Thus denaturation, in this case with distilled water, has again affected the indices of normal calcium exchange.

As a further check on the meaning of the constants given in table 3, it was decided to investigate the effect of leakage through the exposed cut nerve ends. Consequently, one nerve was tied with silk at both ends and treated in the standard fashion. The two slopes obtained, whose values are given in table 4, are seen to lie within the normal range. Consequently, the contribution of the diffusion or exchange through the nerve ends is relatively unimportant, unless the nerves are damaged by tying so that the loss from the tied nerves exactly equals that from the cut ones.

TABLE 5

(a) *Effect of 4 mM/l calcium concentration on Ca^{45} outward flux*

NERVE	$k_1 \times 10^2$/min.	$k_2 \times 10^3$/min.	a/b
151	11.20	2.90	4.95
152	8.67	5.02	2.55
153	7.45	3.95	1.52
154	10.30	5.06	1.34
155	10.40	4.50	1.60
Average	9.60	4.29	2.39
Standard deviation	1.5	0.9	1.5

(b) *Effect of 2 hour preliminary incubation in non-radioactive van Harreveld's solution on Ca^{45} outward flux*

NERVE	$k_1 \times 10^2$/min.	$k_2 \times 10^3$/min.	a/b
166	9.5	4.64	4.06
167	10.6	5.80	5.12
168	11.7	10.4	6.56
169	9.75	4.77	7.63
Average	9.91	6.40	6.84
Standard deviation	1.0	2.7	1.6

Effect of calcium ion concentration. The calcium ion concentration of the bathing fluid was dropped to the lowest value consistent with satisfactory Ca^{45} measurements (4 mM/l) in order to determine the influence of the calcium ion concentration. The nerves tested were exposed to this concentration throughout the period of study, in the incubation period as well as throughout the rinsing and planchet series.

Table 5 (a) lists the results obtained with 4 nerves. The average k_1 and k_2 are within the normal range. Consequently, a calcium ion concentration change of this magnitude has no demonstrable effect on the calcium exchange, as measured by this technique. In view of the high standard deviation of the figures for a/b in the normal control, the low value given in table 5 (a) for this ratio cannot be considered as significant.

TABLE 6

Ca^{45} outward flux at 25°C.

NERVE	$k_1 \times 10^2$/min.	$k_2 \times 10^3$/min.	a/b
3	11.3	1.56	1.38
4	7.03	1.89	1.52
5	7.44	2.45	1.52
Average	8.59	1.97	1.47
Standard deviation	2.35	0.44	0.08

We would expect that Ca leakage from the nerve would increase in an external medium in which the Ca concentration was reduced to one-third of its normal value. The observation that the indices of Ca transport are not affected by this decrease in external Ca lends support to our inference that these indices do, in fact, represent Ca exchange, and are not closely connected to simple Ca leakage.

It has been suggested that the high Ca concentration of van Harreveld's solution may affect the Ca^{45} transport system of the axon. If this were true preliminary incubation in normal van Harreveld's for two hours should markedly affect the normal indices of Ca^{45} outward flux. Accordingly 4 nerves were incubated in the non-radioactive solution for two hours and were then treated in a normal fashion with a one hour incubation in Ca^{45} van Harreveld's and subsequent washing. The results are given in table 5 (b) and are seen to be in reasonable agreement with those obtained in the normal nerve.

As a consequence it may be assumed that the high Ca concentration in van Harreveld's solution does not affect the outward Ca^{45} flux in any appreciable manner under our experimental conditions.

Temperature coefficient. The temperature coefficient of Ca^{45} outward flux was investigated by raising the temperature to 25°C. Table 6 gives the results of experiments with three nerves carried out at this temperature. The average value of k_1 was found to be the same as in the 15°C. experiment, while the value of k_2 appears lower than that at 15°C., though not necessarily significantly so.

If we were dealing with a chemical reaction of the type normally encountered in biological systems, we would expect the reaction rate to double for a 10° temperature rise. On the other hand, if we were dealing with a process of diffusion in a watery medium, we would expect the reaction to proceed about 30% faster for a 10° rise in temperature, as a result of the temperature coefficient of the viscosity of water. Since k_1 does not seem to increase, and since k_2 appears to decrease with increasing temperature, neither of the suggestions above appears to be valid, and the explanation for the anomalous temperature coefficient must await further experiments.

DISCUSSION

The characteristic wash-out curve consisting of the sum of two exponentials is typical of the behavior that might be expected in transport from two compartments (Solomon, '49), as would be the case if some of the calcium were more tightly bound than the rest. In agreement with this supposition, Solomon and Tobias ('51) have tentatively suggested that in dog nerve some Ca is bound as a negatively charged complex. However the characteristic wash-out curve would also describe a system in which some of the calcium were in a part of the nerve separated from the rest of the calcium. As shown in the appendix, the constants k_1 and k_2 do not give directly the transport coefficients (or rate constants) connecting the two separate compartments, but are themselves composite, each containing terms involving all the transport coefficients. Hence the evidence that it is possible to alter k_2 without affecting k_1, as is the case with heat denaturation, cannot be taken as substantiating the concept of the two compartment system. Indeed, in the absence of any supporting evidence that two such compartments exist, it is possible that the double exponential slope of the wash-out curve is purely fortuitous and the constants may bear no relationship to any intra-axonal barriers, either physiological or chemical. Nonetheless, it is desirable to express our results in the double exponential form even in the absence of any physiological explanation of the meaning of the coefficients because it then becomes possible to describe the data in a quantitative fashion.

It seemed worthwhile to see whether the exponential curve obtained could be the result of simple diffusion of Ca from a model nerve conceived as a uniform cylinder, whose length is long compared to its diameter. Jacobs ('35) has shown that diffusion out of such a cylinder would be given by an equation including zero and first order Bessel functions. In these equations the amount of material, R, remaining at time, t, in a cylinder from which it is diffusing is given by an infinite series of exponential terms each with a steeper slope and smaller amplitude than its predecessor. The first two terms are:

$$R = 4\,\mu_o \pi r^2 L \left(\frac{e^{-5.75\,Dt/r^2}}{5.75} + \frac{e^{-30.5\,Dt/r^2}}{30.5} \right) \qquad (2)$$

where μ_o is the initial concentration of Ca^{45}, D is the diffusion constant of Ca^{45}, r is the radius of the cylinder, and L is its length.

To test this equation, an experiment was carried out in which the diffusion of Ca^{45} from a cellophane bag was measured. Such a system is analogous to a nerve of uniform interior composition bounded by a very thin membrane which provides a sudden step from interior to exterior concentration. The curve obtained with the bag consisted of the sum of two exponentials and can be described as:

$$R = a\ [e^{-0.0624t} + 0.41\ e^{-0.0113t}] \qquad (3)$$

In equation (2) the amplitude of the second term compared to the first is 0.19, and the ratio of the exponents is 5.30. In equation (3) the amplitude ratio is 0.41 and the slope ratio is 5.52, in reasonable agreement with equation (2). In the case of the normal nerve, the first ratio is 4.0 and the second is 18.1 as given in table 3. These clearly do not agree with equation (2).

This conclusion may mean either that the simple cellophane bag system does not represent the chela nerve, which is not unlikely in view of the over-simplicity of the model; or that the process of Ca^{45} transport out of the nerve is not simple diffusion. The ancillary evidence supports the second conclusion, since denaturation by heat has been shown to affect the outward flux of Ca^{45} considerably, and since a large change in external Ca concentration does not affect the outward flux.

ACKNOWLEDGMENT

This research has been supported in part by the Office of Naval Research and the Atomic Energy Commission. The authors wish to express their appreciation to Dr. A. B. Hastings and Dr. E. G. Ball for their advice and help and to Mrs. Frances Newton for measuring the radioactivity.

SUMMARY

The change in Ca^{45} content with time at 15°C. of a crayfish chela nerve previously incubated in van Harrevelds' solution is given by:

$$R = b\ (4.0\ e^{-0.0883t} + e^{-0.00487t})$$

where $R = Ca^{45}$ content at time t, and b is an arbitrary constant. Lowering the Ca concentration in the bathing medium to 4 mM/l does not affect the Ca^{45} loss. The temperature coefficient (for a 10° rise to 25°C.) of the process is very small or negative.

APPENDIX

The equations that follow are presented to show that the constants k_1 and k_2 are not simply related to the transfer coefficients in a simple mathematical model. There are two such simple models which can be made to fit a double exponential

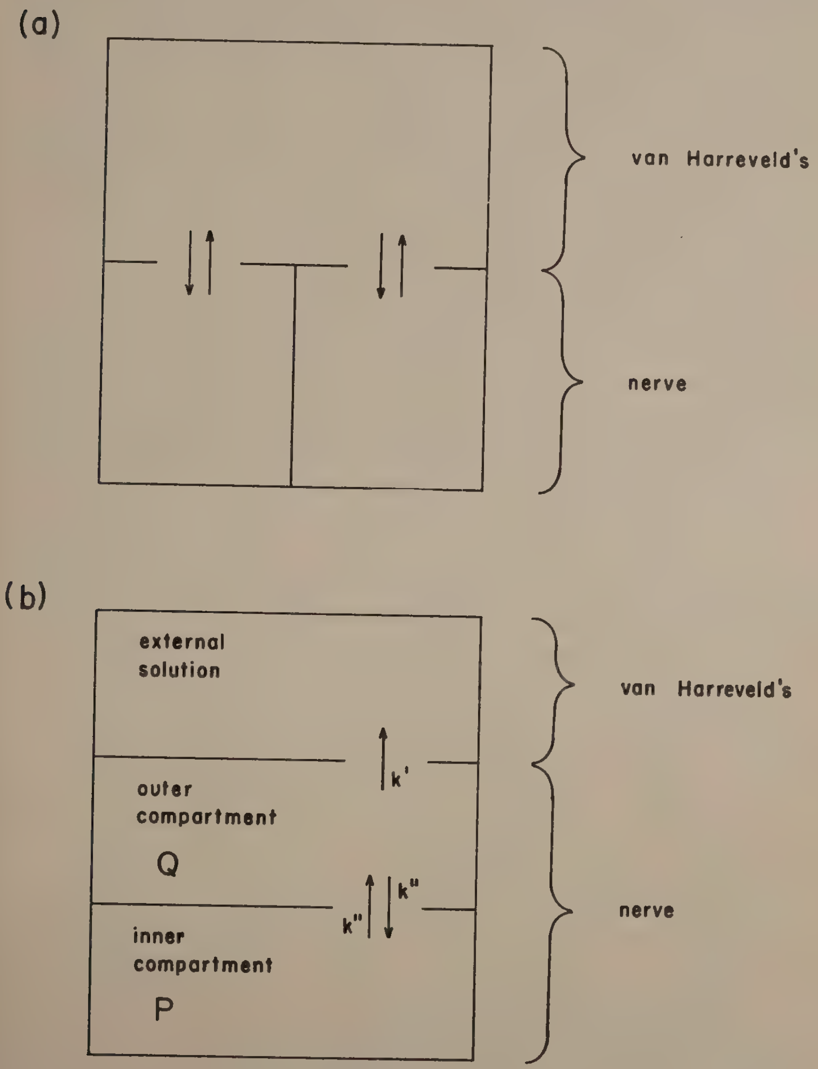


Figure 2

wash-out curve, and they are illustrated in figure 2. We have arbitrarily chosen to use the model of three components in series (fig. 2 b), although a similar set of equations could be derived for the model shown in figure 2 a.

There is no back reaction from the external solution to the outer nerve compartment since the nerves are washed in non-radioactive van Harreveld's solution. We have assumed that the rate constant for the entrance of Ca into the inner compartment is equal to that for the exit, which is the situation that obtains in diffusion. However, since the volumes are unknown, this choice does not introduce a restriction on the system.

The equations follow:

$$dP/dt = k''q - k''p \tag{4}$$

$$dQ/dt = k''p - k''q - k'q \tag{5}$$

where P and Q are the amounts of Ca^{45} in the inner and outer compartments respectively ($P + Q = R$, the amount of Ca^{45} in the nerve), p and q are the relative Ca^{45} concentrations, k'' and k' are transport coefficients as shown in figure 2 b, and t is time. P and Q are related to p and q as follows:

$$\begin{aligned} P &= pv_p \\ Q &= qv_q \end{aligned} \tag{6}$$

where v_p and v_q are the water volumes of the inner and outer compartments respectively. Let us define

$$\begin{aligned} k_{pq} &= k''/v_q \\ k_{qp} &= k''/v_p \\ k_{qo} &= k'/v_q \end{aligned} \tag{7}$$

In the steady state for Ca, the solutions to 4 and 5 are:

$$\begin{aligned} Q &= \alpha e^{-rt} + \beta e^{-st} \\ P + Q &= (1/k_{qp})\ [\alpha s e^{-rt} + \beta r e^{-st}] \end{aligned} \tag{8}$$

where α and β are constants and

$$\begin{aligned} r &= 1/2\left[k_{qp} + k_{qo} + k_{pq} + \sqrt{(k_{qp} - k_{qo})^2 + k_{pq}^2 + 2k_{pq}\ k_{qo} + 2k_{pq}\ k_{qp}}\right] \\ s &= 1/2\left[k_{qp} + k_{qo} + k_{pq} - \sqrt{(k_{qp} - k_{qo})^2 + k_{pq}^2 + 2k_{pq}\ k_{qo} + 2k_{pq}\ k_{qp}}\right] \end{aligned} \tag{9}$$

It can be seen that there is no simple relationship between r and s (which are analogous to k_1 and k_2) and any of the transfer coefficients, either k_{pq}, k_{qp} and k_{qo} or k' and k''.

LITERATURE CITED

van Harreveld, A. 1936 Proc. Soc. Exp. Biol. Med., *34:* 428.

Jacobs, M. H. 1935 Ergeb. der Biologie, *12:* 114.

National Bureau of Standards Circular 499. Nuclear Data.

Rothenberg, M. A. 1950 Biochemica Biophysica Acta, *4:* 96.

Solomon, A. K. 1949 J. Clin. Inv., *28:* 1297.

Solomon, S., and J. M. Tobias 1951 Biol. Bull., *101:* 198.

A COMPARATIVE STUDY OF RENAL FUNCTION IN MARINE TELEOSTS[1]

ROY P. FORSTER[2]
The Department of Zoology, Dartmouth College, Hanover, New Hampshire,
and The Mount Desert Island Biological Laboratory,
Salisbury Cove, Maine

THREE FIGURES

Studies on marine fish have played a central role in the initiation and development of current concepts concerning kidney function (Marshall, '34). Fish are of particular interest to the renal physiologist because of the variety of nephron units found within this group and because of the dramatic nature of the water and salt-balance situations which are derived from the intimate relationship between fish and their aquatic environment. These comparative studies, utilizing modern clearance techniques, were undertaken to delineate the specific functions of glomeruli and renal tubules when the fish were maintained under normal conditions and also when they were exposed to a variety of stress states.

The waters of Frenchman Bay provide a unique opportunity for kidney function studies involving glomerulo-tubular balance because of the availability of three fish with similar and relatively undifferentiated renal tubules which are associated in varying degrees with glomerular filtering devices.

The goosefish, Lophius piscatorius, is completely aglomerular (Marshall and Grafflin, '28; Grafflin, '29). Its blood supply to the kidney is almost entirely venous. The renal tubules have an essentially uniform epithelium and show only the

---

[1] This work was supported in part by a grant from the Rockefeller Foundation.

[2] Fellow of the John Simon Guggenheim Memorial Foundation during 1949 when many of the studies included in this report were made.

structural differentiation characteristic of the proximal convolutions typically present in the freshwater fish and higher vertebrates.

The common longhorn sculpin, Myoxocephalus octodecimspinosus, has kidneys which are completely glomerular. Careful examination has disclosed no blind or aglomerular tubules (Nash, '31). The renal tubule consists of only one segment, ending in a very short collecting duct. Marshall and Grafflin ('32) refer to this segmentally undifferentiated tubule as a proximal tubule, homologous with the renal tubule of aglomerular fishes and with the proximal segment of the mammalian nephron. The glomerular circulation is arterial. The renal tubules have a double supply of blood, as do all mesonephric kidneys, the first from the efferent glomerular arterioles and a second from the venous renal-portal system.

In the shorthorn or daddy sculpin, Myoxocephalus scorpius, the renal tubules are predominantly aglomerular, and indeed, Grafflin ('33) has shown that some specimens appear functionally to be entirely aglomerular. Grafflin noted, however, that even those fish showing no functional glomerular activity, when examined histologically, exhibited numerous glomerular structures with degenerative features. These degenerative changes, which involved both the vascular tuft and the neck segment, seemed most marked in the larger specimens. Grafflin speculated that this fish in its lifetime undergoes the glomerular degeneration which marine fish have exhibited during the course of evolution as an adaptation for water conservation necessitated by the hypertonic nature of their external environment. However, this view needs modification in the light of data to be described here.

PROCEDURES

Both species of sculpins were obtained by handline off the dock of the Mount Desert Island Biological Laboratory, where only one shorthorn or daddy sculpin appeared for every 25 or 50 longhorns taken. Large shorthorn sculpins are quite sluggish and were sometimes taken with a dip net after being spotted motionless on the bottom in still water at low tide.[3] The goosefish were obtained by dragging an otter trawl at a depth of 35 fathoms along the east shore of Long Porcupine and Round Porcupine Islands near the mouth of Frenchman Bay. After capture the fish were maintained either in floating live cars or in laboratory aquaria supplied with running sea water.

All injections and the collections of blood and urine were made without removing the fish from water. The animals were transferred in dip dishes, and a specially designed trough was used to immobilize the fish during experimental procedures without exposing the gills. Urine was usually collected by indwelling catheters which were plugged during the clearance periods, allowing the urine to accumulate in the bladder, and unplugged at collection times. For clearance studies the bladder was washed several times, especially when urine flows were low. When only one clearance period was sought in an animal (longhorn sculpins) the bladder was emptied by stroking the abdomen, the urogenital papilla tied shut, and at the end of the period the body cavity was opened and urine collected by allowing it to flow directly from the punctured bladder into weighed vials. This method is not recommended for shorthorn sculpins because of the difficulty encountered in completely emptying the bladder by applying pressure on the abdomen.

Blood collections were made at the midpoint or at the beginning and end of clearance periods. A 25 gauge needle was inserted into the fleshy part of the tail on the ventral side in the midline posterior to the urogenital papilla. After the resistance of the vertebral column was encountered the needle was withdrawn slightly and suction applied. Blood can be drawn very quickly by this method, particularly if the bevel of the needle is turned to face in the direction of blood flow and care is taken to keep the gills immersed. There seems to be a respiratory-cardiac synchrony in fish which makes it difficult to collect blood quickly when opercular movements have ceased, as when the animals are out of water.

---

[3] The distinguishing features of the shorthorn sculpin are as follows: the spines are relatively blunt, the upper cheek-bone never reaches more than halfway to the edge of the gill cover, the two dorsal fins are of the same height, and the belly tends to be colored reddish-orange with large round white spots in the male and yellowish spots in the female (Bigelow and Welsh, '24).

Injections were sometimes made via the caudal vessels which permitted starting the clearance measurements as soon as a reasonable time had been allowed for mixing, but usually clearance measuring substances were injected intraperitoneally, and the quantitative collection of urine begun only after 24 hours or so had elapsed, with experimental observations being made while plasma concentrations were slowly falling.

Inulin clearances were used to measure glomerular filtration rates (Clarke, '36), and p-aminohippurate clearances for the measurement of minimal renal plasma flow (Chasis et al., '45). As in mammals, PAH clearances in fish were found invariably to exceed simultaneous glomerular filtration rates, they were self-depressed at high plasma concentrations of PAH, and they were independent of plasma concentrations under 5 mg %.

Inulin concentrations in urine and plasma were determined by Roe's resorcinol method as modified by Schreiner ('50), and PAH after the method described by Smith et al. ('45). Sodium was measured against an internal lithium standard with a Perkin-Elmer flame photometer, chloride after the method of Van Slyke and Hiller ('47) and freezing point depressions through the use of a "thermistor" (Western Electric Co.) in which resistance measurements were related to a standard NaCl curve corrected for activity.

RESULTS AND DISCUSSION

Physiological evaluation of glomerular activity. Previously it has been shown that inulin is not excreted by the aglomerular kidneys of goosefish (Shannon, '34), toadfish (Richards et al., '34; and Shannon, '34), or batfish (Smith, '37), and the evidence on other species gathered to date indicates that this polysaccharide is neither secreted nor reabsorbed by the renal tubules of glomerular nephrons (see Smith, '51, for review). Hence, one can assume that its clearance measures the filtration rate in such partially glomerular kidneys as those of the shorthorn or daddy sculpin (Myoxocephalus scorpius). Grafflin ('33) has made a histological examination of a series of 16 shorthorn sculpins varying in weight from 85 to 1006 gm

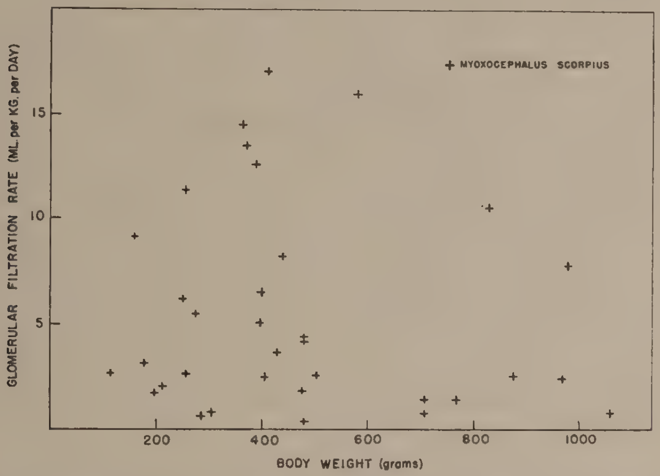


Fig. 1 Glomerular filtration rates in a series of 35 shorthorn or daddy sculpins (Myoxocephalus scorpius) showing the absence of a direct relationship between the age or size of the fish and the glomerular filtration rate.

and reported evidence of glomerular degeneration in all. Even the youngest fish had large numbers of shrunken glomeruli with completely avascular tufts, and the associated tubules had markedly constricted neck segments. The fraction of what appeared to be functionally patent glomeruli diminished with the size of the fish, and in the largest fish it was practically impossible to find any glomeruli which appeared functional. It was concluded that in this form a progressive glomerular degeneration occurred during the lifetime of the fish.

To test the hypothesis that glomeruli degenerate with age or size inulin clearances were used to measure the filtration rate in a series of 34 shorthorn sculpins varying in weight from 116 to 1060 gm. As can be seen in figure 1 no direct correspondence was observed between the size of the fish and the filtration rate. In 5 of the fish only traces of inulin could be detected in urine when simultaneous plasma concentrations were as high as 200 mg %. These fish, weighing 282, 304, 479, and 1060 gm could be considered essentially aglomerular (glomerular filtration rates in diuresis were less than 1 ml per kilogram per day). Filtration rates in all were considerably lower than in the completely glomerular longhorn sculpin as will be seen subsequently. Most of the shorthorn sculpins which were not excessively diuretic had filtration rates of less than 3 ml per kilogram per day, while longhorn sculpins in this range of urine flow had inulin clearances varying between 15 and 35 ml per kilogram per day. Under these circumstances the shorthorn sculpins were filtering only 1 ml out of each liter of plasma delivered to the kidneys as determined by the PAH clearance. There was some tendency for filtration rates to rise with increased urine flows but, on the other hand, several of the most nearly aglomerular fish exhibited the highest urine flows.

Recruitment of glomerular function during "laboratory diuresis." It has been repeatedly noted that marine fish traumatized in capture, by retention for long periods in live cars or aquaria, or by manipulations required to carry on renal clearance studies, invariably develop a diuresis which is associated with corresponding increases in filtration rate (Grafflin, '31; Clarke, '34; and Pitts, '34). Current studies indicate that this diuresis is not limited to fish with glomerular kidneys (fig. 2). In the shorthorn sculpin urine flows increased from 2 ml to 40 ml per kilogram per day in fish that were essentially aglomerular as judged by the excretion of inulin (fig. 2). A similar diuresis occurs in the completely aglomerular goosefish where urine flows as high as 75 ml per kilogram per day were noted in animals maintained in the laboratory. The osmotic changes occurring in this diuresis will be described in a later section of this paper.

With minimal handling Clarke ('36) noted xylose clearances (80% of inulin clearance) which averaged 13.6 ml per kilogram per day and urine flows averaging 3.3 ml. In the experiments reported here (fig. 2) no unusual efforts were made to duplicate "normal" conditions, but at low urine flows glomerular filtration rates were observed as low as 12 ml per kilogram per day and averaged less than 20. With high urine flows glomerular filtration rates averaged 100 ml per kilogram per day, and rates as high as 188 ml per kilogram per day have been noted. As Smith ('51) has suggested this faculty of increasing glomerular filtration rates 10-fold or more indi-

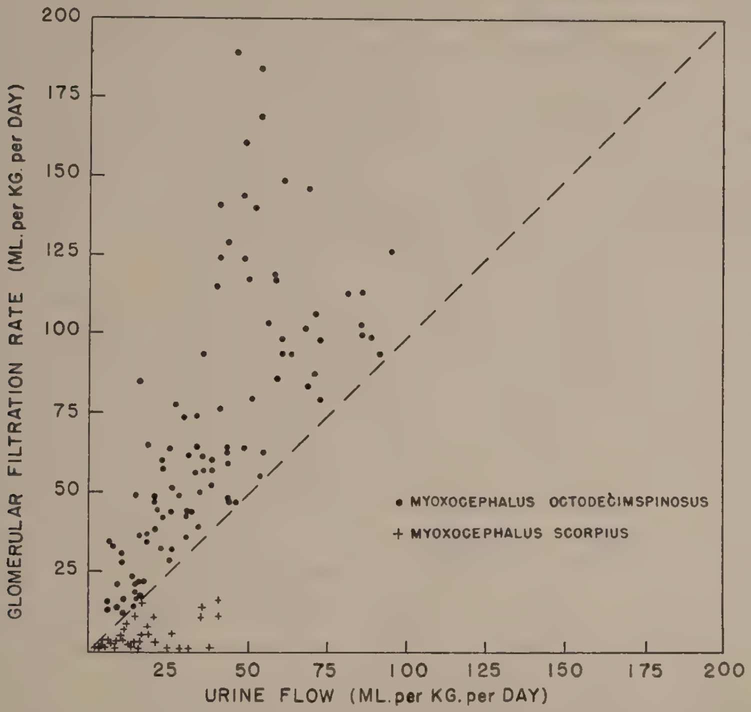


Fig. 2 Relationship of glomerular filtration rates to the increased urine flows accompanying "laboratory diuresis" in the completely glomerular longhorn sculpin (M. octodecimspinosus) and in the partially glomerular shorthorn or daddy sculpin (M. scorpius). Each item represents an individual fish.

cates that some factor normally operates to diminish glomerular activity, and that under the circumstances prevailing during diuresis the effect of this factor is negated, effecting thereby a maximal recruitment of glomerular function. This is reminiscent of changes in glomerular filtration recorded in frogs (Forster, '38 and '42) and in freshwater turtles (Friedlich, Holman and Forster, '40), except that in these forms the filtration rate increases because free water is available, whereas in the marine fishes, the increase in filtration rate apparently reflects a breakdown of some active inhibitory process. The tremendous increase in glomerular filtration rate which accompanies retention in captivity and handling suggests that in their normal state marine fish essentially are physiologically aglomerular.

W. H. Sawyer and M. K. Sawyer ('52) have again demonstrated in amphibians and reptiles that constriction of the afferent glomerular arteriole during antidiuresis can be effected by the injection of posterior pituitary extracts. Burgess, Harvey and Marshall ('33) noted that such injections into the fresh water catfish (Ameiurus nebulosus) do not alter urine flow. The net effect of the antidiuretic hormone, whether it acts via skin, afferent arteriole or distal tubule, is always to increase the total water content of the body. Fresh water fish might be expected to be independent of such antidiuretic control because the need to conserve water never arises, due to their markedly hypotonic external environment. However, marine fish in hypertonic sea water are physiologically in the same situation waterwise as amphibians out of water, and one might expect that an antidiuretic factor operates similarly to limit filtration rates and conserve water, whereas such a factor may be absent in fresh water fish.

Marine elasmobranchs such as the dogfish do not exhibit a spontaneous "laboratory diuresis." However, Clarke ('36) has shown that urine flows in dogfish could be increased by placing them in 70% sea water which resulted in inulin clearances as high as 272 ml per kilogram per day as compared with a control value with the fish in sea water of 90 ml per kilogram per day. In frogs, the only other cold-blooded form extensively studied, glomerular filtration rates approach one liter per kilogram per day during maximal diuresis (Forster, '43).

Renal plasma flow measurements. The clearance of p-aminohippurate at low plasma levels was used to measure renal plasma flows. As in the other classes of vertebrates, PAH

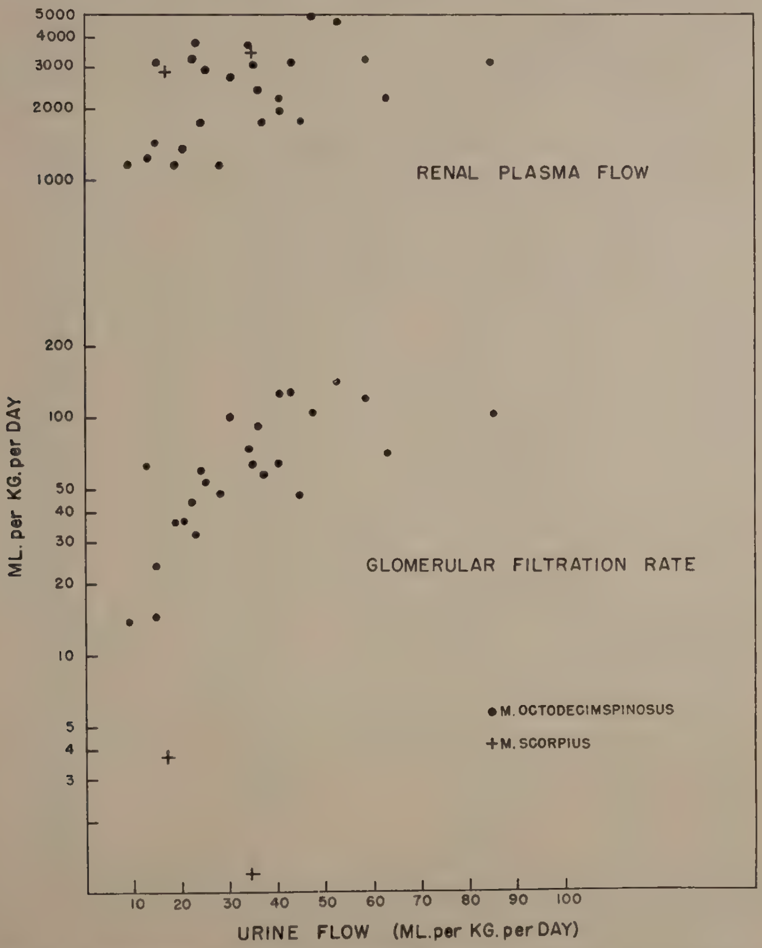


Fig. 3 A semilogarithmic plot showing the relationship of renal plasma flow and glomerular filtration rate to urine flow in 25 different longhorn sculpins and in two partially glomerular shorthorn sculpins.

was found to possess the characteristics of plasma flow measuring substances inasmuch as it was eliminated at a very rapid rate; at low plasma levels its clearance was independent of the concentration in plasma; and as the plasma PAH levels were raised the PAH clearances were depressed. It is not

TABLE 1

Filtration fractions in a series of sculpins grouped according to urine volumes

URINE FLOW	GLOMERULAR FILTRATION RATE	RENAL PLASMA FLOW	FILTRATION FRACTION
	ml/kg/day		
Longhorn sculpins			
9.3	14.0	1185	.012
14.5	14.5	1760	.008
14.7	23.8	3120	.008
18.5	36.2	1110	.032
20.5	37.7	1369	.028
22.1	44.6	3260	.014
22.7	32.3	3880	.008
24.2	60.0	1760	.034
25.6	64.5	2900	.022
28.2	48.0	1180	.041
30.2	100.1	2700	.037
33.8	74.0	3720	.020
Average 22.0	Average 45.7	Average 2323	Average .0241
34.8	64.5	3040	.021
36.0	93.0	2450	.038
36.8	57.0	1760	.032
40.4	64.1	1910	.034
40.5	124.0	2190	.057
43.0	129.0	3140	.041
44.5	46.5	1740	.027
52.5	140.6	4620	.030
57.5	103.0	4930	.021
58.5	118.0	3240	.036
63.5	70.0	2190	.032
85.6	103.6	3040	.034
Average 49.5	Average 92.7	Average 2860	Average .0360
Shorthorn sculpins			
16.8	3.7	2860	.0013
34.5	1.2	3540	.0003

practicable to determine directly the fraction of PAH extracted by fish kidneys, hence, the PAH clearances must be regarded merely as representing minimal renal plasma flows.

Table 1 is a summary of all observations made on fish when plasma concentrations of PAH were between 1 and 6 mg %. Twenty-four longhorn sculpins were studied with urine flows ranging from 9.3 to 85.6 ml per kilogram per day, and two shorthorn sculpins with urine flows of 16.8 and 34.5 respectively. Renal plasma flows seem to range between one and 5 liters per kilogram per day. The renal plasma flow apparently is related to the concomitant urine flow and glomerular filtration rate (fig. 3). The 12 longhorn sculpins with higher urine flows (34.8–85.6 ml per kilogram per day) had glomerular filtration rates which averaged twice as high as those of the group with lower urine flows. However, the concomitant renal plasma flows in this markedly diuretic group averaged only 19% higher.

The fraction of renal plasma flow filtered at the glomeruli is very small, even in the completely glomerular longhorn sculpins. In the low urine flow group 24 ml on the average is filtered out of each liter of plasma delivered to the kidneys, and in the group with higher urine flows this value rises to 36. In one of the shorthorn sculpins 3.7 ml of glomerular filtrate was formed when the plasma flow was 2860 ml per kilogram per day, and in the other only 1.2 ml were filtered out of 3540. These very small filtration fractions emphasize the relative insignificance of the glomerular circulation in marine fish. The renal portal circulation completely sustains urinary excretion in the goosefish and almost completely in the shorthorn sculpin. In even the completely glomerular longhorn sculpins the arterial circulation, as judged by the glomerular filtration rates, seems to comprise only a small fraction of the total renal blood flow. In the frog, supplied also with a renal portal circulation, more than 6% of the renal plasma flow is filtered at the glomerulus (Forster, '43).

PAH Tm in the glomerular undifferentiated tubule. No studies previously have been made to determine whether a maximal limit exists with respect to the amount of substrate which can be transported per unit time in the undifferentiated renal tubule of the glomerular fish. Such a tubular maximum (Tm) has been shown to characterize secretion in aglomerular kidneys (Shannon, '38), and in the differentiated tubules of amphibians (Forster, '40), birds and mammals (Smith, '51, for review). Large amounts of PAH were injected into a series of 14 fish, and plasma concentrations were obtained ranging from 20 to 296 mg %. The rate of PAH secretion was found to be independent of the concentration of PAH in plasma and averaged 147 mg per kilogram per day. Six observations made with plasma concentrations between 20 and 50 mg % averaged 131 mg per kilogram per day, two between 80 and 100 mg % averaged 149, three between 120 and 140 mg % averaged 140, two between 140 and 160 mg % averaged 163 and 7 observations made with plasma concentrations over 160 mg % averaged 152 mg per kilogram body weight per day. Direct ultrafiltration measurements through cellophane disclosed that an insignificant quantity of PAH was bound to plasma protein. Hence, measurement of Tm was made by merely subtracting the quantity filtered (product of inulin clearance and amount of PAH in 1 ml of plasma) from the total amount excreted. It is clear that the maximal limit which is imposed upon tubular transport in the aglomerular fish and in the differentiated renal tubule of the higher vertebrates also characterizes transport in this glomerular undifferentiated renal tubule.

Minimal renal plasma flow calculations were made when PAH plasma concentrations were noted within the range of 3 and 6 mg % at the midpoint of the urine collection period. At these low plasma levels the rate of PAH secretion was well below Tm, and PAH clearances were independent of simultaneous plasma concentrations. No attempt was made to determine the actual plasma concentration at which saturation of the secretory mechanism occurred because of the impracticability of making multiple clearance observations on these small fish, and because of the difficulty involved in comparing individual fish whose physiological states showed wide variations.

The maximal rate of tubular excretion of PAH noted in 4 observations on one goosefish averaged 137 mg per kilogram per day, and single observations on three shorthorn sculpins averaged 138 mg per kilogram per day, which figures are comparable with the value of 147 found in the longhorn sculpin.

The self-depression of PAH secretion with high PAH plasma concentrations noted in mammals (Eggleton and Habib, '50, and Schachter and Freinkel, '51) was not observed in these fish. Longhorn sculpins with plasma concentrations of 290 mg % secreted PAH at the rate of 149 mg per kilogram per day (average $Tm_{PAH} = 147$), and at the highest plasma concentration observed in goosefish, 138 mg %, the rate was 168 mg per kilogram per day (average = 137).

Water excretion by renal tubules. Longhorn sculpins maintained for three days in sea water diluted to approximate isotonicity exhibited progressive diuresis, but the filtration rates fell in time until finally urine volumes greatly exceeded simultaneous filtration rates. Five such longhorn sculpins were found to have urine flows of 58.4, 43.6, 25.0, 22.9 and 34.5 ml per kilogram per day when simultaneous inulin clearances were 38.9, 19.7, 13.0, 11.6 and 4.9 respectively. It is unlikely that the depressed inulin clearances were due to the back diffusion of inulin because under these conditions plasma inulin concentrations were significantly higher than

TABLE 2

Urine volumes and filtration rates in a longhorn sculpin maintained in isotonic sea water for a prolonged period

TIME	URINE FLOW	INULIN CONC.		INULIN U/P	FILTRATION RATE
		Plasma	Urine		
	ml/kg/day	*mg %*			*ml/kg/day*
24 hrs.	29.2	108	280	2.60	75.7
96 hrs.	58.4	36	24	0.67	38.9

those in urine. Whatever the mechanism may be, it is clear that under certain conditions the completely glomerular renal tubules of longhorn sculpins can add water to the glomerular filtrate.[4]

Extra-renal excretion. Some substances, such as phenol red and PAH, which are secreted by the renal tubules of fish are also actively removed from the blood stream by the liver. Following intravenous injection, phenol red can be directly observed in a highly concentrated solution in the gall bladder, and the intestinal contents are invariably colored. Quantitative recoveries of 4 mg of intravenously administered PAH in three longhorn sculpins, each weighing about 200 gm, showed that within 4 hours 50% appeared as free PAH in the urine and 15% in the bile and residual intestinal fluids. A single observation on a goosefish disclosed a PAH concentration of 1005 mg % in bile when the simultaneous plasma concentration was 141.

Effect of cold on renal function. Eighteen longhorn sculpins were maintained in refrigerated aquaria at 7°C. and 18 control animals were kept at 17°C., under identical conditions with respect to aeration and light, to test the effect of temperature on glomerular function and PAH transport by the tubules at high PAH plasma levels. These fish were found to be cold-adapted, and capable of vigorous activity upon stimulation, down to temperatures slightly below 0°C.

Filtration rates were unaffected by this 10° temperature variation. The rate of PAH transport by the tubules, however, showed a temperature coefficient of about 2.0. The renal tubules of fish maintained at 7°C. transported PAH at the rate of 69.5 mg per kilogram per day, and those kept at 17°C. at the rate of 138.6 mg per kilogram per day.

---

[4] The finding that longhorn sculpins maintained in isotonic sea water from the time of capture were found to undergo the customary diuresis within 24 hours demonstrates that the diuretic process could not have been initiated by osmotic water loss to a hypertonic environment subsequent to skin traumatization, which has been suggested by Smith ('32) and Grafflin and Ennis ('34) as the initiating event in this diuretic disease. Urine flows of 29.2, 10.9 and 13.4 ml per kilogram per day were noted in three fish in which simultaneous inulin clearances were 75.7, 39.4 and 62.8 ml per kilogram per day respectively. There is little doubt that the diuretic state is invariably accompanied by ingestion of sea water.

Osmotic pressure relations in normal animals. Table 3 presents the freezing point and chloride data on blood and urine samples obtained from fish immediately after capture. The osmotic pressure difference noted between plasma and urine averaged 15%, somewhat more than 0.1°C. or one atmosphere. Diuresis ensued almost immediately after capture and under such conditions the osmotic pressure difference between plasma and urine narrowed. This was demonstrated in an experiment where 4 longhorn sculpins had their urogenital papillae tied immediately upon capture and were then placed in a live car for 12 hours to allow urine to accumulate. Blood was taken at the end of this period and it was found that the urine: plasma freezing point ratio had risen to 0.94, 0.89, 0.96 and 0.94 (average 0.934), in contrast to an average of 0.85 for freshly caught fish.

The chloride data presented in table 3 again emphasize the previously observed fact that urine samples obtained from freshly caught fish are essentially chloride-free, while simultaneous plasma concentrations under normal conditions average 180 m. eq. per L. in these fish (Grollman, '29; Grafflin, '31; and Pitts, '34). There is also considerably less Na in urine than in simultaneously collected plasma. Analyses on two goosefish showed plasma concentrations of 179 and 119 mg per L. while the urine samples contained 64.5 and 6.5 mg per L. respectively.

In large goosefish 15–20 ml of pericardial fluid can be readily obtained. Table 4 presents the data on the freezing point depressions of these fluids compared with simultaneously collected plasma and urine in three freshly caught goosefish. The urine is consistently more dilute than plasma and is roughly isotonic with pericardial and coelomic fluid. Unlike urine, pericardial and coelomic fluids have chloride and sodium concentrations only slightly lower than simultaneously collected plasma. Chloride concentrations in the pericardial fluids of the three fish represented in table 4 were 158, 158.5,

TABLE 3

Chloride and freezing point data on blood and urine samples obtained from fish immediately after capture

ANIMAL		WEIGHT	HEMATOCRIT	PLASMA		URINE		FREEZING POINT U/P
				Chloride	Freezing point	Chloride	Freezing point	
		kg	*% R.B.C*	*m.eq./L*	*°C.*	*m.eq./L*	*°C.*	
Shorthorn	1	.32	23	182	—.706	8.8	—.580	0.82
Shorthorn	2	.20	18	187	—.706	9.3	—.622	0.88
Longhorn	1	.14	18	183	—.715	21.0	—.622	0.87
Goosefish	1	11.40	14	174	—.655	12.1	—.570	0.87
Goosefish	2	9.10	22	176	—.670	13.6	—.545	0.81
Goosefish	3	4.50	19	180	—.670	12.3	—.570	0.85

and 163 m. eq. per L. when simultaneous plasma concentrations were 174, 176, and 180. In the single determination on coelomic fluid the chloride concentration was 166, with the simultaneous pericardial fluid 162 m. eq. per L.

These observations on osmotic pressures of body fluids not only show that the kidneys of marine teleosts play no role in the regulation of osmotic pressure but indicate that they add to, rather than alleviate, osmotic difficulties by forming urine more dilute than plasma. That simultaneously collected pericardial and coelomic fluid was found to be hypotonic to plasma, and to approximately the same extent as urine, gives rise to the unlikely possibility that the interstitial fluid in the kidney is more dilute in electrolyte than blood. Alternate explanations are equally awkward, namely, that the aglomerular tubule actively secretes water independently of electrolyte, or that the undifferentiated tubule permits water to move passively toward the lumen following electrolyte secretion in one region and then specifically reabsorbs salt and not water at some distal point.

TABLE 4

Comparative freezing points of body fluids obtained from freshly captured aglomerular goosefish, expressed in °C.

ANIMAL	PERICARDIAL FLUID	COELOMIC FLUID	URINE	PLASMA
Goosefish 1	—.580		—.570	—.655
Goosefish 2	—.555		—.545	—.670
Goosefish 3	—.610	—.620	—.570	—.670

Osmotic pressure changes during "laboratory diuresis." Table 5 is a summary of the freezing point and chloride observations made on urine and plasma collected from a goosefish which was already in a characteristically diuretic state at the start of the collections, as attested by the high chloride content of the urine as well as by the high urine flow. It is interesting to note that at the time of the first blood collection the chloride content of the plasma was in the range of "normal'' animals (actually lower than in the three fish represented in table 4). However, in the 53 hours intervening between the two blood collections the plasma chloride content rose from 168 to 199 m. eq. per L. Chloride concentrations in the urine exceeded simultaneous plasma concentrations throughout the diuretic state.

Urine volume steadily increased following capture, reached a peak, and then diminished just before death. Chloride concentrations in urine paralleled rates of flow except at both ends of the diuresis. Grafflin ('31) has shown that in longhorn sculpins urine flows may increase from 1 up to 15 ml per kilogram per day with the urine remaining essentially chloride-free. Again, after the peak of diuresis, urine flow fell markedly but the chloride concentration continued to climb. This fall in urine flow just before death is a constant feature of the diuretic state and has been pointed out by both Grafflin ('31) and Pitts ('34). Chloride concentrations in urine which exceed simultaneous plasma concentrations are characteristic of highly diuretic fish.

TABLE 5

Urine volumes, chloride excretion and freezing point observations made on plasma and urine taken from an aglomerular goosefish during the course of spontaneous ''laboratory diuresis''

SAMPLE	TIME	VOLUME	CHLORIDE Concentration	CHLORIDE Amount excreted	FREEZING POINT
	hours	*ml/kg/day*	*m.eq./L*	*m.eq./kg/day*	°C.
Plasma	17		168		—.610
Urine no. 1	0–17	29.4	180	5.29	—.610
Urine no. 2	17–23	38.4	199	7.64	—.625
Urine no. 3	23–31	54.8	208	11.40	—.650
Urine no. 4	31–39	65.0	222	14.43	—.680
Urine no. 5	39–47	75.0	232	17.40	—.720
Urine no. 6	47–53	67.3	242	16.29	—.760
Plasma	53		199		—.775

The total osmotic concentrations of both plasma and urine increased steadily during diuresis and remained essentially isotonic throughout. A freezing point depression difference of 0.15°C. was noted between the first and last urine periods during which time the chloride content rose from 180 to 242 m. eq. per L. With the handling involved in these experiments the normal 15% difference between freezing point depressions in urine and plasma fell to zero within 24 hours. Merely collecting urine overnight with the urogenital papillae tied reduced the difference to 6.6% when the accumulated urine was compared to plasma taken at the end of the period. Pericardial fluid taken at the end of the experiment had a freezing point of — .74°C. and the chloride content was 215 m. eq. per L.

The sequence of events taking place during the spontaneous diuresis characteristic of marine teleosts in captivity is by no means completely clear. It can perhaps be assumed that increased ingestion and intestinal absorption of sea water accompanies it, inasmuch as there are no other possible avenues of entry for the large volumes of fluid known to be excreted by the kidneys. While the accumulation of univalent ions in body fluids may possibly be due to an interference with the specific gill secretory mechanism, it is more likely that this also results from increased intestinal uptake, especially because Mg and SO_4 which are excreted exclusively by the kidney (Smith, '30) pile up too, and are eliminated in progressively greater amounts as the diuretic state proceeds (Pitts, '34). Smith showed that the forcible administration of sea water enhances intestinal absorption, and Grafflin and Ennis ('34) that obstruction of the intestinal tract results in a marked shutdown in urine formation.

Accommodation to the influx of water which results from increased intestinal absorption is partially achieved by increased urine flows in both glomerular and aglomerular fish, but the very low hematocrits noted during diuresis (frequently less than 10%) indicate that the adjustment is not complete. The increased rate of salt absorption presumably taxes to the limit the gill's specific secretory capacity, and after the renal tubules have accommodated to the extent of failing to exercise their prerogative of retaining univalent ions, no further effective adjustments can be made, and the animal's body fluids become progressively flooded with salt. Normally the kidneys are actually a liability as far as osmotic regulation is concerned, but, though they do not take on a specific regulatory function under the osmotic stress imposed by this diuretic state, at least they do not contribute to the difficulty by persisting in the old hypotonic habits which perhaps were acquired during their early evolution as teleosts living in fresh water. The excess Mg and SO_4 absorbed may act as tubular diuretic agents (Bieter, '31 and '35), but even the excretion of copious volumes of isotonic urine fails to keep up with the rate at which hypertonic fluid is absorbed from the intestine.

What should cause marine teleosts to drink copious quantities of sea water after capture is not at all clear. Further study is needed to throw light on the genesis of diuresis, as well as on other problems raised by these investigations, such as the nature of the factors underlying recruitment of glomerular activity during diuresis and the mechanisms involved in the secretion of water and salt by renal tubules.

CONCLUSIONS

1. While in their natural state, glomerular and aglomerular marine teleosts form urine which is significantly hypotonic to plasma, with an osmotic pressure difference of approximately one atmosphere. The urine is essentially Cl-free and low in Na. Pericardial and coelomic fluids are likewise hypotonic to plasma. During the course of "laboratory diuresis" the osmotic pressure of plasma rises, monovalent ions appear in high concentration in urine, and finally, urine and plasma become isotonic and remain so while the solute concentrations of both increase throughout the progressively diuretic state.

2. Renal plasma flows in marine teleosts range between one and 5 liters per kilogram body weight per day. The relative unimportance of the glomerular circulation, as compared with that of higher vertebrates, is illustrated by the very small filtration fractions noted in even such completely glomerular forms as the longhorn sculpin.

3. Shorthorn or daddy sculpins (Myoxocephalus scorpius) have very low rates of glomerular filtration compared with the totally glomerular longhorn sculpins (M. octodecimspinosus), and indeed some appear to be physiologically aglomerular. However, they do not exhibit progressive reductions in filtration rate with increasing age or size.

4. The undifferentiated renal tubule of the glomerular longhorn sculpin is characterized by a maximal limit with respect to the amount of p-aminohippurate which can be transported per unit time, similar to that imposed on aglomerular tubules and the differentiated renal tubule of other vertebrates. No self-depression of Tm_{PAH} at high plasma PAH levels was noted in these marine teleosts.

5. Under certain conditions the ability to excrete water was exhibited by the renal tubules of completely glomerular fish as well as by the partially glomerular and totally aglomerular forms. Longhorn sculpins maintained for long periods in isotonic or slightly hypotonic sea water had urine flows significantly higher than simultaneous glomerular filtration rates.

6. Extra-renal excretion of PAH and phenol red is accomplished by the liver in marine teleosts.

7. Sculpins are cold-adapted forms to the extent that they are capable of vigorous activity, even when environmental temperatures approach 0°C. Glomerular filtration rates are unaffected by low temperatures. The temperature coefficient for Tm_{PAH} is 2.0.

ACKNOWLEDGMENTS

J. F. Lucey collaborated in many of the experiments conducted during the summers of 1949 and 1950. E. C. Matthews assisted with the development of techniques for handling the fish during the summer of 1948. J. C. Scott-Baker, G. A. Zak, E. L. Becker and L. B. Page cooperated in the determinations of chloride, sodium and freezing point depressions. I am grateful to all of the above for their assistance.

LITERATURE CITED

Bieter, R. N. 1931 The action of some diuretics upon the aglomerular kidney. J. Pharm. and Exp. Therap., *43:* 399.

——— 1935 The action of diuretics injected into one kidney of the aglomerular toadfish. J. Pharm. and Exp. Therap., *53:* 347.

Bigelow, W. B., and W. W. Welsh 1924 Fishes of the Gulf of Maine. Bull. U. S. Bureau of Fisheries, *40:* 320.

Burgess, W. W., A. M. Harvey and E. K. Marshall, Jr. 1933 The site of the antidiuretic action of pituitary extract. J. Pharm. and Exp. Therap., *49:* 237.

Chasis, H., J. Redish, W. Goldring, H. Ranges and H. Smith 1945 The use of sodium p-aminohippurate for the functional evaluation of the human kidney. J. Clin. Invest., *24:* 583.

Clarke, R. W. 1934 The xylose clearance of Myoxocephalus octodecimspinosus under normal and diuretic conditions. J. Cell. and Comp. Physiol., *5:* 73.

——— 1936 Simultaneous xylose and inulin clearances in the sculpin. Bull. Mt. Desert Isl. Biol. Lab., 25.

——— 1936 Experimental production of diuresis in the dogfish. Bull. Mt. Desert Isl. Biol. Lab., 25.

Eggleton, M. G., and Y. A. Habib 1950 Excretion of para-aminohippurate by the kidney of the cat. J. Physiol., *110:* 458.

Forster, R. P. 1938 The use of inulin and creatinine as glomerular filtrate measuring substances in the frog. J. Cell. and Comp. Physiol., *12:* 213.

——— 1940 A renal clearance analysis of phenol red elimination in the frog. J. Cell. and Comp. Physiol., *16:* 113.

——— 1942 The nature of the glucose reabsorptive process in the frog renal tubule. Evidence for intermittency of glomerular function in the intact animal. J. Cell. and Comp. Physiol., *20:* 55.

——— 1943 The effect of epinephrine upon frog renal hemodynamics in the intact animal. Am. J. Physiol., *140:* 221.

Friedlich, A., C. B. Holman and R. P. Forster 1940 Renal clearance studies in the fresh-water turtle, Pseudemys elegans. Bull. Mt. Desert Isl. Biol. Lab., 24.

Grafflin, A. L. 1929 The pseudoglomeruli of the kidney of Lophius piscatorius. Am. J. Anat., *44:* 441.

——— 1931 Urine flow and diuresis in marine teleosts. Am. J. Physiol., *97:* 602.

——— 1933 Glomerular degeneration in the kidney of the daddy sculpin (Myoxocephalus scorpius). Anat. Rec., *57:* 59.

Grafflin, A. L., and D. Ennis 1934 The effect of blockage of the gastrointestinal tract upon urine formation in a marine teleost, Myoxocephalus octodecimspinosus. J. Cell. and Comp. Physiol., *4:* 283.

Grollman, A. 1929 The urine of the goosefish (Lophius piscatorius): its nitrogenous constituents with special reference to the presence in it of trimethylamine oxide. J. Biol. Chem., *81:* 267.

Marshall, E. K., Jr. 1934 The comparative physiology of the kidney in relation to theories of renal secretion. Physiol. Rev., *14:* 133.

MARSHALL, E. K., JR., AND A. L. GRAFFLIN 1928 The structure and function of the kidney of Lophius piscatorius. Bull. Johns Hopkins Hosp., *43:* 205.

——————— 1932 The function of the proximal convoluted segment of the renal tubule. J. Cell. and Comp. Physiol., *1:* 161.

NASH, J. 1931 The number and size of glomeruli in the kidneys of fishes with observations on the morphology of the renal tubules of fishes. Am. J. Anat., *47:* 425.

PITTS, R. G. 1934 Urinary composition in marine fish. J. Cell. and Comp. Physiol., *4:* 389.

RICHARDS, A. N., B. B. WESTFALL AND P. A. BOTT 1934 Renal excretion of inulin, creatinine, and xylose in normal dogs. Proc. Soc. Exp. Biol. and Med., *32:* 73.

SAWYER, W. H., AND M. K. SAWYER 1952 Adaptive responses to neurohypophyseal fractions in vertebrates. Physiol. Zool., *25:* 84.

SCHACHTER, D., AND N. FREINKEL 1951 Self-depression of Tm_{PAH} in the dog at high plasma PAH levels and its reversibility by acetate. Am. J. Physiol., *167:* 531.

SCHREINER, G. 1950 Determination of inulin by means of resorcinol. Proc. Soc. Exp. Biol. and Med., *74:* 117.

SHANNON, J. A. 1934 The excretion of inulin by the dogfish, Squalus acanthias. J. Cell. and Comp. Physiol., *5:* 301.

——————— 1938 The renal excretion of phenol red by the aglomerular fishes Opsanus tau and Lophius piscatorius. J. Cell. and Comp. Physiol., *11:* 315.

——————— 1938 Renal excretion of exogenous creatinine in the aglomerular toadfish, Opsanus tau. Proc. Soc. Exp. Biol. and Med., *38:* 245.

SMITH, H. W. 1929 The composition of the body fluids of the goosefish (Lophius piscatorius). J. Biol. Chem., *82:* 71.

——————— 1930 The absorption and excretion of water and salts by marine teleosts. Am. J. Physiol., *93:* 480.

——————— 1932 Water regulation and its evolution in the fishes. Quart. Rev. Biol., *7:* 1.

——————— 1937 The physiology of the kidney. Oxford University Press.

——————— 1951 The kidney. Oxford University Press.

SMITH, H. W., N. FINKELSTEIN, L. ALIMINOSA, B. CRAWFORD AND M. GRABER 1945 The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man. J. Clin. Invest., *24:* 388.

VAN SLYKE, D. D., AND A. HILLER 1947 Application of Sendroy's iodometric chloride titration to protein containing fluids. J. Biol. Chem., *167:* 107.

NOTICE TO CONTRIBUTORS

THE JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY, appearing bimonthly, is intended as a medium for the publication of papers which embody the results of original research of a quantitative or analytical nature in general and comparative physiology, including both their physical and chemical aspects. Short preliminary notices are not desired and papers will not be accepted for simultaneous publication or which have been previously published elsewhere. While not specifically excluding any particular branch of physiology, contributors should recognize that excellent journals already exist for publication in the field of experimental and physiological zoology, dealing particularly with genetics, growth, behavior, developmental mechanics, sex determination, and hormonal interrelationships, and also for pure mammalian functional physiology and the physical chemistry of non-living systems. Preference will be given to analyses of fundamental physiological phenomena whether the material is vertebrate or invertebrate, plant or animal. Since the journal is restricted, it is not possible to publish more than a limited number of papers which must be short and concise.

It is recognized that prompt publication is essential, and the aim will be to issue papers within three months of acceptance.

Manuscripts and drawings should be sent to the Managing Editor, DETLEV W. BRONK, Office of the President, The Johns Hopkins University, Baltimore 18, Maryland.

The paper must be accompanied by an author's abstract **not to exceed 225 words in length,** which will appear on the advance abstract cards of the Bibliographic Service of The Wistar Institute in advance of complete publication. Nothing can be done with the manuscript until the abstract is received.

Manuscripts should be typewritten in double spacing on one side of paper 8½ × 11 inches, and should be packed flat — not rolled or folded. The original, not carbon, copy should be sent. The original drawings, not photographs of drawings, should accompany the manuscript. Authors should indicate on the manuscript the approximate position of text figures.

Manuscripts and drawings should be submitted in complete and finished form with the author's complete address. All drawings should be marked with the author's name. The Wistar Institute reserves the privilege of returning to the author for revision approved manuscript and illustrations which are not in proper finished form for the printer. When the amount of tabular and illustrative material is judged to be excessive, or unusually expensive, authors may be requested to pay the excess cost.

The tables, quotations (extracts of over five lines), and all other subsidiary matter usually set in type smaller than the text, should be typewritten on separate sheets and placed with the text in correct sequence. Footnotes should not be in with the text (reference numbers only), but typewritten continuously on separate sheets, and numbered consecutively. Explanations of figures should be treated in the same manner, and, like footnotes, should be put at the end of the text copy. A condensed title for running page headlines, not to exceed thirty-five letters and spaces, should be given.

Figures should be drawn for reproduction as line or halftone engravings, unless the author is prepared to defray the additional cost of a more expensive form of illustration. All colored plates are printed separately and cost extra. In grouping the drawings it should be borne in mind that, after the reduction has been made, text figures are not to exceed the dimensions of the printed matter on the page, 4¼ × 6¾ inches. Single plates may be 5 × 7½ inches, or less, and double plates (folded in the middle), 11½ × 7½ inches. Avoid placing figures across the fold, if possible.

Figures should be numbered from 1 up, beginning with the text figures and continuing through the plates. The reduction desired should be clearly indicated on the margin of the drawing.

All drawings intended for photographic reproduction either as line engravings (black-ink pen lines and dots) or halftone plates (wash and brush work) should be made on white or blue-white paper or bristol board — not on cream-white or yellow-tone. Photographs intended for halftone reproduction should be securely mounted with colorless paste — never with glue, which discolors the photograph.

Galley proofs and engraver's proofs of figures are sent to the author. All corrections should be clearly marked thereon.

The journal furnishes the author fifty reprints, with covers, of the paper gratis. Additional copies may be obtained according to rates which will be sent the author as soon as the manuscript has been examined at The Wistar Institute, after acceptance.

THIS NUMBER COMPLETES VOLUME 42

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

VOL. 42 DECEMBER 1953 No. 3

CONTENTS

H. M. PATT, S. H. MAYER, R. L. STRAUBE AND E. M. JACKSON. Radiation dose reduction by cysteine. Six figures 327

A. L. BENNETT, F. WARE, JR., A. L. DUNN AND A. R. MCINTYRE. The normal membrane resting potential of mammalian skeletal muscle measured in vivo. Two figures 343

GEORGE E. DANIEL AND HELEN D. PARK. Glutathione and x-ray injury in Hydra and Paramecium 359

J. WENDELL BURGER AND CHEVES MCC. SMYTHE. The general form of circulation in the lobster, Homarus. Thirteen figures 369

KARLMAN WASSERMAN, E. LOVELL BECKER AND ALFRED P. FISHMAN. Transport of phenol red in the flounder renal tubule. One figure 385

A. GLENN RICHARDS, MARION B. CLAUSEN AND MYRTLE N. SMITH. Studies on arthropod cuticle. X. The asymmetrical penetration of water. Eight figures 395

ROBERT BALLENTINE. The biosynthesis of stable cobalto-proteins by plants. II. Interaction of iron and cobalt metabolism in neurospora crassa. Four figures 415

HOWARD P. JENERICK. Muscle membrane potential, resistance, and external potassium chloride. Six figures 427

MORGAN HARRIS AND PHYLLIS B. KUTSKY. Utilization of added sugars by chick heart fibroblasts in dialyzed media. Five figures 449

SIDNEY SOLOWAY, J. H. WELSH AND A. K. SOLOMON. Studies on Ca^{45} transport in crayfish nerve. Two figures 471

ROY P. FORSTER. A comparative study of renal function in marine teleosts. Three figures 487

PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA

Printed in the United States of America